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A "biomarker+histopathology" toolbox in *Solea* spp. for the assessment of the biological effects of pollution in the context of the EU Marine Strategy Framework Directive

Tifanie Briaudeau

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Department of Zoology and Animal Cell Biology
Research Group: Cell Biology in Environmental Toxicology

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I. General Introduction

1. Environmental protection of the marine and coastal ecosystems

1.1. The anthropogenic pressure

Concerns on environmental protection first emerged half a century ago and was strongly focused on human interest. The United Nations Conference on the Human Environment declared in 1972:

"*Man has the fundamental right to freedom, equality and adequate conditions of life, in an environment of a quality that permits a life of dignity and well-being, and he bears a solemn responsibility to protect and improve the environment for present and future generations*."

The consciousness for marine environment pollution was already present in this declaration with the Principle 7:

"*States shall take all possible steps to prevent pollution of the seas by substances that are liable to create hazards to human health, to harm living resources and marine life, to damage amenities or to interfere with other legitimate uses of the sea*."

Indeed, the aquatic environment has always attracted humans' interest for its diverse advantages such as fishing activities, water transportation, offshore oil and gas exploitation, among others (Figure 1). Coastal and estuarine areas are particularly used as a strategic point for communication through navigation, for industrial development (e.g. metallurgy, smelters, paper milling, oil refineries, shipbuilding and power stations), agricultural activities and recreation (tourism, housing, water sports). Inevitably, the extensive use of marine resources and the extreme settlement of humans along the coastline have generated a high anthropogenic pressure on the aquatic ecosystem putting at risk its survival and the viability of its resources (Islam and Tanaka, 2004; Law et al., 2010; Pan and Wang, 2012). Discharge wastewaters reach the aquatic environment and

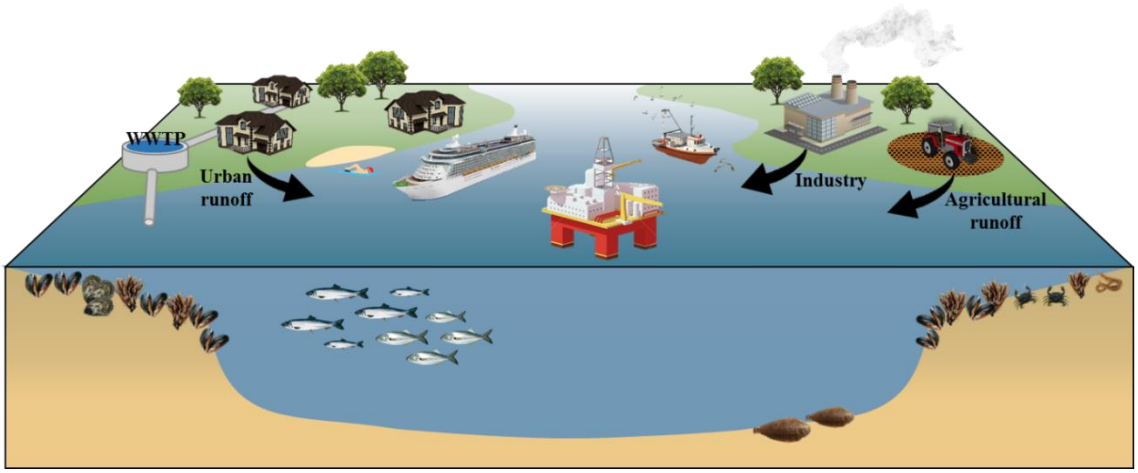


Figure 1. Main anthropogenic activities along estuarine and coastal areas and subsequent sources of hazardous substances reaching the aquatic ecosystem. Scheme modified from OSPAR, 2010).

tend to accumulate in confined areas such as estuaries (Law et al., 2010). These areas are also victims of dredging activities, essential for water transportation but at the origin of habitat deterioration (Martins et al., 2012). Coastal and estuarine environments are particularly crucial for the marine ecosystem as they serve as nursery grounds for many marine species (Le Pape et al., 2007). Thus, the decline in estuarine ecosystem quality generated by pollution loading and habitat loss may affect species and species recruitment level and lead to severe consequences on the survival of the biota.

1.2. Awareness and actions

In response to the clear deterioration of marine environmental conditions, legislative measures and policies have been taken to protect the aquatic ecosystem. In the European Union, the Water Framework Directive (WFD, 2000/60/EC) provides a list of chemicals described as priority substances to be surveyed to reach "good ecological status". This implies that contaminants found in the ecosystem (water, sediment and biota) are at levels not leading to harmful effects in aquatic organisms. Likewise, the Marine Strategy Framework Directive (MSFD, 2008/56/EC) aims to ensure that *"concentrations of contaminants are at levels not giving rise to pollution effects*" in both coastal and offshore environments (Law et al., 2010). To do so, the concentration of contaminants in the environment and the measurement of biological parameters in relation with pollutants effects are used together to assess the general health status of the aquatic ecosystem. The protection of the European marine environment is also under the implementation of Regional Sea Conventions (RSCs): OSPAR (North-East Atlantic), HELCOM (Baltic Sea), Barcelona Convention (coastal region of the Mediterranean Sea) and Bucharest Convention (Black Sea). These international legal requirements provide strategies to prevent environmental pollution and preserve aquatic ecosystems. In the case of the North-East Atlantic, the Joint Assessment and Monitoring Programme (JAMP) and the International Council for the Exploration of the Sea (ICES) guide the assessment of the general health status of the aquatic environment under the OSPAR Commission. They include the regulation of priority chemical substances, the development of appropriate environmental management and guidelines to assess the general status of the marine environment with the implementation of monitoring programmes (Hylland et al., 2017a).

1.3. From chemical analysis to biological effects

Monitoring programmes were first developed to prevent contaminants consumption for human health. Programmes were based on chemical analysis for the detection and quantification of pollutants in the different compartments of the aquatic environment (water column, sediment and biota). The Mussel Watch Program was one of the first biomonitoring programmes to assess contaminants accumulation and concentrations in aquatic organisms such as mussels and oysters, based on chemical analysis (Farrington et al., 1983; Goldberg et al., 1983; Martin, 1985). Later on, the development of biological approaches allowed for the assessment of biological effects of this chemical contamination (Bayne, 1989). Thus, biological parameters were used in parallel with chemical analysis to detect and quantify contaminants in the environment and to assess

their impact on the ecosystem (Gray, 1992; OSPAR, 1998). Nowadays, most environmental risk assessments integrate both chemical and biological monitoring of contaminants, which include the identification and quantification of pollutants and their effects on the surrounding biota (OSPAR, 2000, 2008; Davies and Vethaak, 2012; HELCOM, 2018; Hylland et al., 2017a, 2017b; Vethaak et al., 2017).

Biological effects of environmental stressors (e.g. exposure to pollutants, changes in temperature, low food availability) are assessed in selected marine organisms called sentinel species (Beeby, 2001). At first, sentinel species were used for their capacity to accumulate contaminants in order to simply assess the chemicals bioavailability in the ecosystem (Philips and Segar, 1986). However, sentinel species are also characterised by a wide but specific geographical distribution, they are abundant, they have a well-documented biological cycle and they are sensitive to pollutants and suitable to life in captivity and to laboratory conditions (Widdows, 1985; Cajaraville et al., 2000; Beeby, 2001). The Mussel Watch Program (Goldberg et al., 1983) was one of the first biomonitoring programmes that used marine organisms (mussels and oysters) as sentinel species to assess environmental concentrations of pollutants. Together with molluscs, teleosts (e.g. *Gadus morhua*, *Merluccius merluccius*, *Mullus* sp.) are also considered target organisms due to their abundance, diversity, ecological and economical relevance, availability and morphological similarity with other vertebrate models (De La Torre et al., 2005; Marigómez et al., 2006; Viarengo et al., 2007; Hylland et al., 2017a, 2017b). In Europe, they have been included in several national and international biomonitoring programmes (MEDPOL-UNEP Mediterranean Biomonitoring Program, OSPAR Convention, RAMOGE; Thain et al., 2008; Law et al., 2010; Lyons et al., 2010, 2017; Burgeot et al., 2017; Vethaak et al., 2017).

In particular, flatfish have attracted great interest as sentinel species for the assessment of the general health status of coastal and estuarine ecosystems (Köhler et al., 1992; Myers et al., 1994; Stehr et al., 2003; Einsporn et al., 2005; Dabrowska et al., 2012; Fricke et al., 2012). Due to their benthic behaviour, their health status is closely related to sediment contamination (Feist et al., 2004; Lang et al., 2006; Jimenez-Tenorio et al., 2008; Costa et al., 2013; Solé et al., 2016). In Northern Europe, dab (*Limanda limanda*), European flounder (*Platichthys flesus*), olive flounder (*Paralichthys olivaceus*) and English sole (*Parophrys vetulus*) are the main flatfish species used for biomonitoring programmes in different regional areas (Köhler et al., 1992; Myers et al., 1994; Vethaak et al., 1996; Myers et al., 2003; Stehr et al., 2003; Stentiford et al., 2003; Lang et al., 2006). These species are less abundant along the Atlantic Iberian coast, which is an inconvenient for their use as sentinels in biomonitoring programmes in this region. Instead, the common sole (*Solea solea*) and the Senegalese sole (*Solea senegalensis*) are two very common flatfish species in the Iberian Peninsula (Figure 2; Quincoces et al., 2011). They are found in sandy-muddy bottoms, where they scavenge the sediment to feed on small benthic invertebrates such as polychaetes, amphipods and bivalves. Soles are also of ecological and economical importance due to their great potential for fish farming (Dinis et al., 1999; Imsland et al., 2003). The extensive and intensive culture of sole in France, Italy, Portugal

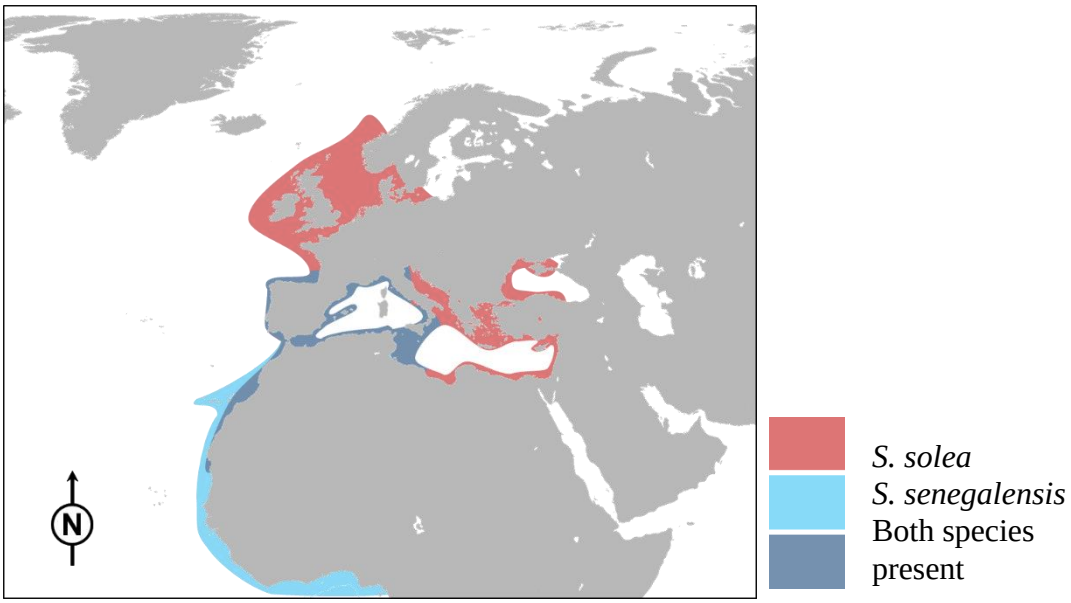


Figure 2. Geographic distribution of *Solea solea* and *Solea senegalensis* in the Eastern Atlantic and the Mediterranean Sea (Desoutter, 1990, 1992).

and Spain has generated a growing interest in the scientific community with great advances in the study of the life cycle, reproduction and culture conditions (e.g. diet development, density adaptation) of various sole species. This knowledge has largely contributed to the use of sole as either sentinel species or model assay organism in toxicological researches, as below detailed.

2. *Solea* spp. as sentinel species for the assessment of coastal and estuarine ecosystem health status

2.1. Biology and ecology of *Solea* spp.

The geographic distribution of the two common sole species found along the Eastern Atlantic coasts differs in latitude. *S. solea* (Quensel, 1806) can be found from Norway to the North coast of Africa, including the Mediterranean Sea, whereas *S. senegalensis* (Kaup, 1858) is present from Senegal to La Rochelle and in the Western Mediterranean Sea (Figure 2; Lagardère et al., 1979; Rodriguez and Rodriguez, 1980; Quéro et al., 1986; Quéro and Vayne, 1997). Both species are found in sandy-muddy bottoms along coastal areas. *S. senegalensis* is well adapted to warm climate and can reach areas up to 100 m depth whilst *S. solea* can be found up to 200 m depth (Whitehead et al., 1986; Durieux et al., 2007). Their feeding activity is mainly nocturnal, peaking at dawn and dusk, and is strongly influenced by olfactory substances (Marchand, 1991). Amongst them, the glycine-betaine is a component of polychaetes, mollusc and crustaceans, which constitute the main elements of sole diet (Molinero et al., 1991). Feeding conditions of flatfish strongly influences their adaptation from larval pelagic stage to benthic behaviour (Marchand, 1991). Sole reproduction is temperature-dependent (>7ºC for *S. solea* and from 13 to 23ºC for *S. senegalensis*) and different geographic areas show different spawning grounds and season. In the North Sea, the breeding period for *S. solea* occurs

in spring in coastal and estuarine areas whilst in the Bay of Biscay, spawning occurs in late winter and beginning of spring, 50 to 80 km from the coast (Laffargue, 2004; Le Pape et al., 2007). In the case of *S. senegalensis*, the main spawning period occurs from February to June with a secondary peak in autumn (Cabral, 2003; Anguis and Cañavate, 2005). Once the eggs have hatched, pelagic larvae are dispersed around the spawning grounds and adopt a circadian vertical migration. The duration of this period is controlled by temperature and can last from 25 to 40 days. Pelagic larvae initiate the metamorphosis whilst migrating towards coastal and estuarine areas where they concentrate closer to the bottom and slowly adopt a benthic behaviour (Figure 3; Marchand, 1991; Lagardère et al., 1999; Amara et al., 2000). As the spawning period of *S. senegalensis* is spread on a long duration, the production of larvae occurs as batches and newly metamorphosed juvenile *S. senegalensis* reach coastal and estuarine areas in different cohorts (Rodríguez Martínez, 1984; Andrade, 1992; Cabral, 2000). Nursery grounds for sole are shallow bays and estuaries (<20 m depth) characterised by high food availability, optimal growth conditions and low predation (Haedrich, 1983; Cabral et al., 2007; Vinagre et al., 2009). The distribution of sole throughout nurseries is influenced by environmental factors such as temperature, salinity, depth, nature of the sediment, food availability and predation (Durieux et al., 2007; Le Pape et al., 2007). Typically, newly metamorphosed benthic larvae (12 mm) are found in upstream areas (muddy bottom and <5 m depth), young juveniles (1 to 6 cm) are present throughout the estuary and older individuals or young adults (7 to 24 cm) concentrate in the outer part of the estuary, bays and coastal areas (Figure 3; Cabral and Costa, 1999; Primo, 2013). Once they become sexually mature, soles migrate out of estuarine areas to reach the deeper spawning grounds (Laffargue, 2004; Primo, 2013). Sexual maturity occurs at similar age (2-4 yr) and size (25-30 cm) for *S. solea*. and *S. senegalensis* (Rogers, 1989; Dinis et al., 1999; Durieux et al., 2007; Vinagre, 2007).

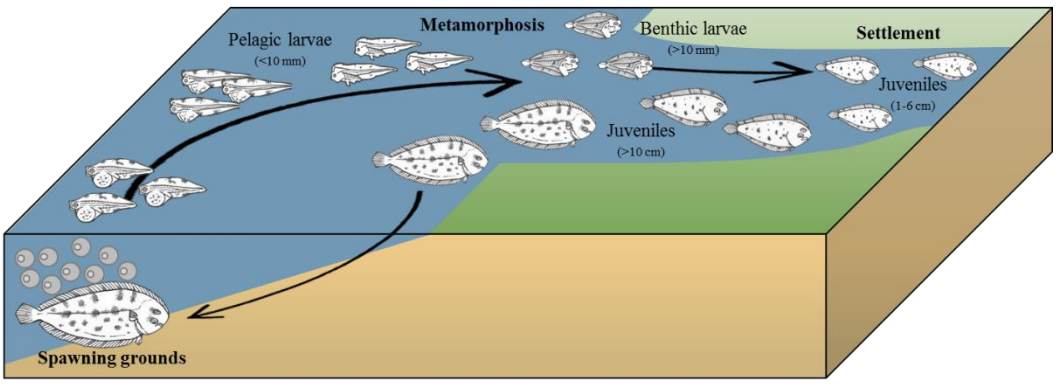


Figure 3. Life cycle of *Solea* spp. in the Iberian Peninsula and the Bay of Biscay.

2.2. Anatomy and physiology of *Solea* spp.

S. solea and *S. senegalensis* belong to the order of pleuronectiformes and are characterised by an asymmetric morphology that they acquire after metamorphosis. During this process, the left eye migrates to the right side, which corresponds to the pigmented upper ocular side. *S. solea* and *S. senegalensis* are morphologically very similar (Figure 4 A-B). They can be distinguished by observation of the pectoral fin of the eyed-side; in the case of *S. solea*, the margin of the fin is coloured with a black spot whilst for *S. senegalensis*, the interradial membrane on the pectoral fin appears black (Figure 4 A-B).

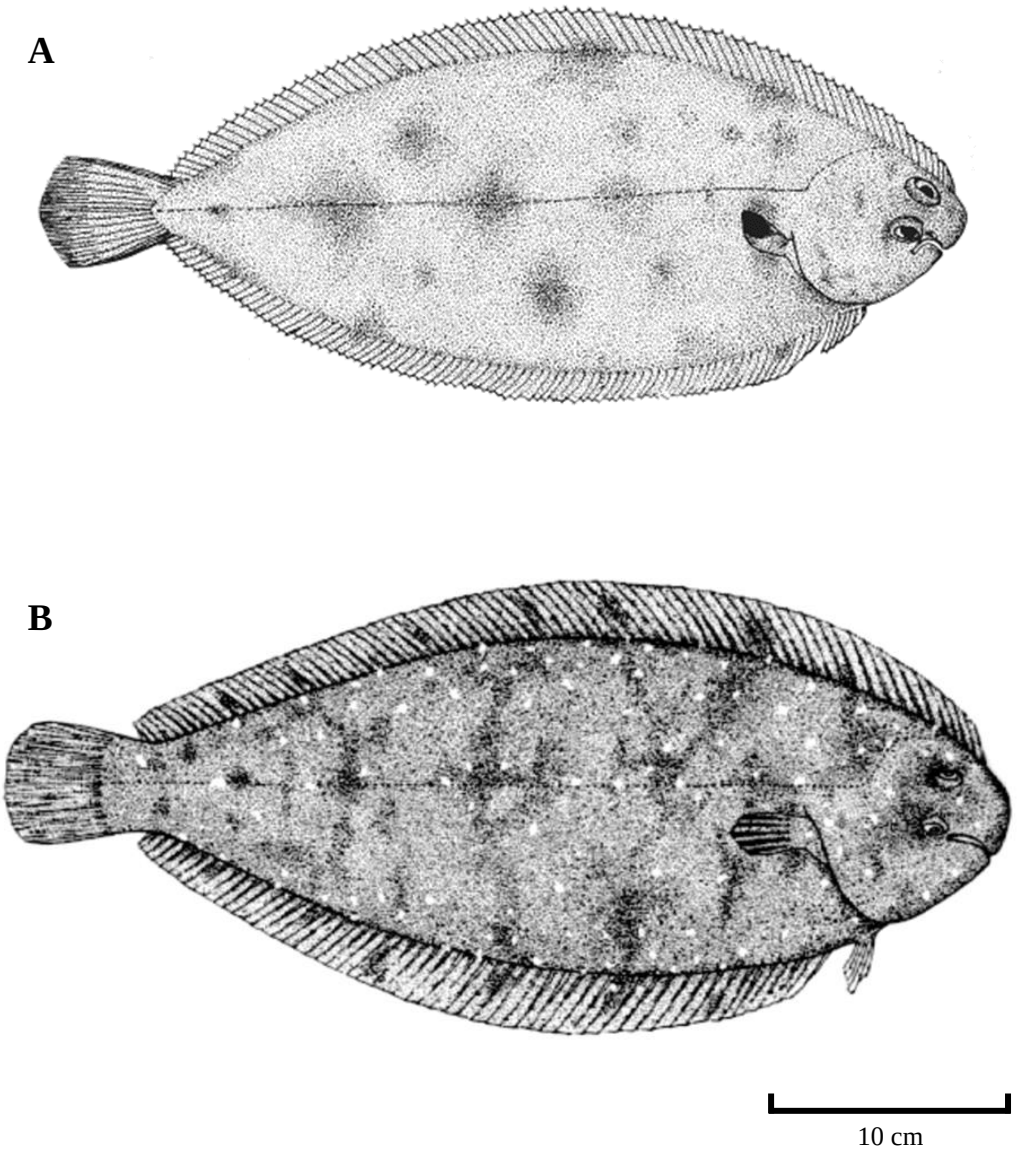
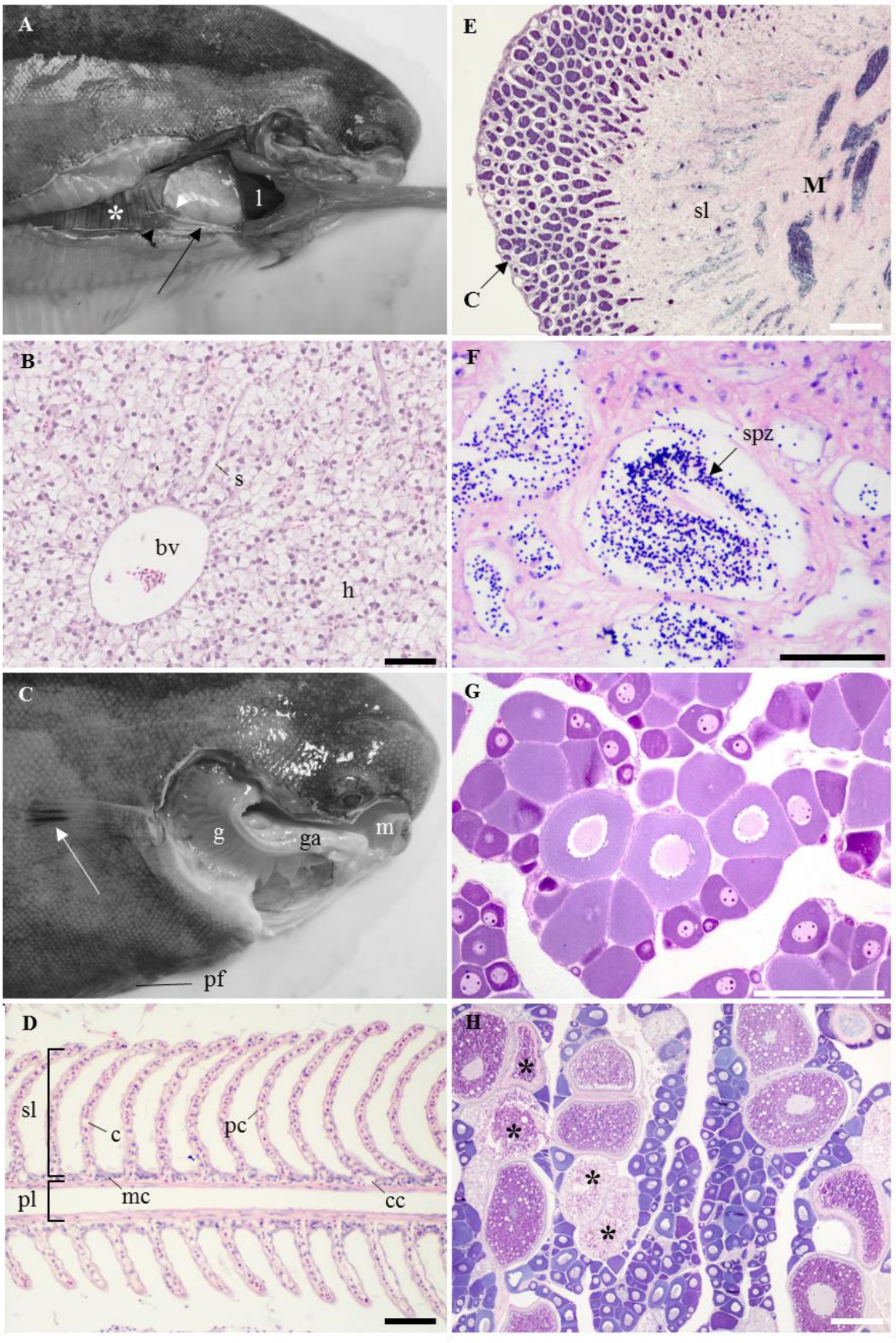


Figure 4. Illustration of *S. solea* (A) and *S. senegalensis* (B) showing the distinct colouration of the pectoral fin (Source: fao.org).

Apart from the evident completion of the metamorphosis, the transition from larvae to juvenile stage is also characterised by the development of the digestive system. It is composed of an oesophagus, a small stomach, a pyloric sphincter and a long intestine. In fact, the ratio stomach length to intestine length is typically small in Soleidae species (Yúfera and Darías, 2007). The liver, gall bladder and pancreas also appear soon in the larval period (Boulhic and Gabaudan, 1992). The liver of sole, like most teleost, has a tubular architecture and is mainly composed of hepatocytes (the parenchyma) arranged in a bi-layer (Hardman et al., 2007; Figure 5 A-B). The general appearance of hepatocytes varied from eosinophilic to clear cytoplasm, depending on the nature and quantity of material stored. Sinusoids and larger vessels separate the tubule units and are connected to the portal vein. The apical membranes of hepatocytes form the tubule lumen where the bile is secreted (Hardman et al., 2007). Bile ducts are lined with a cuboidal epithelium and surrounded by connective tissue. Unlike mammals, the liver of sole also contains the exocrine pancreas. Melanomacrophage centres composed of pigmented cells may also be identified throughout the tissue (Zorita and Cuevas, 2014).

An anatomical review of fish gills is available from Wilson and Laurent (2002). Gills are composed of pairs of arches of osseous structure from which radiate the primary lamellae, supported by cartilage, and the secondary lamellae, originating perpendicularly from the filaments (Figure 5C). The primary lamella is mainly composed of a squamous epithelium, blood sinuses and pillar cells (Figure 5D). The interlamellar space contains pavement, mucous and chloride cells (Figure 5D). The latter are essential for respiration, osmoregulation and to protect the individual from disease, parasites and pathogens. Both mucous and chloride cells are sensitive to environmental changes such as exposure to contaminants (Arellano et al., 2004; Alvarado et al., 2006; Costa et al., 2010a; Martins et al., 2015; Macirella and Brunelli, 2017). The secondary lamellae arise from the primary filaments and are composed of a simple epithelium where direct gas exchanges with the environment occur (Lujić et al., 2013). This thin epithelium is supported by pillar cells inserted between lamellar blood vessels.

Solea spp. males are oligospermic and are characterised by a low gonadosomatic index, as typically observed in flatfish (García-López et al., 2005). Gonads are made of two testicular lobes with two main areas: the cortex with seminiferous lobules and the medulla, which contains the spermatic ducts where the sperm is being collected and stored (Figure 5E). The spermatogenesis is initiated by mitosis where spermatogonial stem cells become spermatogonia (SPG). During the meiosis, SPG are differentiated into spermatocytes (SPC) which evolve into spermatids (SPD). Unlike most flatfish species, the spermatogenesis in *Solea* spp. is asynchronous and semi-cystic: the differentiation of spermatocytes (SPC) and spermatids (SPD) into spermatozoa (SPZ) occurs in the seminiferous lumen (García-López et al., 2005, 2006a, 2006b; Cerdà et al., 2008; Figure 5F). The histological identification of gamete developmental stages is based on the presence and frequency of each type of germ cells in the gonads (García-López et al., 2006a).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The ovary is a paired organ composed of follicles that contain oogonia, which will develop into oocytes. The organ also comprises the stroma (supporting tissue) and vascular and nervous tissues (Blazer, 2002). Females follow a group synchronous ovarian development, which is characterised by the production of at least two populations of oocytes at the same time with one group of larger developing oocytes and another one composed of pre-vitellogenic or resting oocytes (Blazer, 2002; Murua and Saborido-Rey, 2003; Figure 5 G-H). This strategy explains how female soles can produce several batches of mature eggs during a same spawning period (Agulleiro, 2008). The oogenesis is initiated by the cellular proliferation of germ cells. After the complete mitosis of oogonia, the meiosis is stopped at the first prophase and oocyte growth is initiated. This phase is characterised by the accumulation of the hepatic protein, the vitellogenin, in the ooplasm and draws the beginning of the vitellogenesis. In *S. senegalensis*, oocytes grow from 50 µm when immature to more than 500 µm before maturation. It is during the maturation stage that the oocyte nuclear membrane breaks and the meiosis continues until the second metaphase and will not be completed until fertilisation. If not ovulated, a mature oocyte will initiate the phagocytic process of atresia (Figure 5H). Atretic oocytes present a fragmented zona radiata and are highly vacuolated and hypertrophied. It is a natural event that is most commonly detected in post-spawning period, although it can be observed at any stage of the reproductive cycle (Agulleiro, 2008).

In females, sexual maturity can be assessed by external appearance with the detection of abdominal swelling caused by the increase in size of mature gonads (García-López et al., 2006b). This technique is efficient for culture purposes but does not permit to distinguish the different gamete developmental stages to assess the reproduction cycle of wild animals for biomonitoring programmes. Instead, gamete developmental stages are identified by histological observation and can be classified into four categories, based on the frequency of each oocyte phase detected in the gonads (Murua and Motos, 2006).

Figure 5. Anatomy of *S. senegalensis* (A-C) and normal histology of the liver (B), gills (D), male gonads (E-F), pre-vitellogenic female gonads (G) and late vitellogenic female gonad (H). White asterisk: central skeletal portion overlapping the kidney; l: liver; black arrow head: ocular side testicular lobe; white arrow head: blind side testicular lobe; black arrow: spermatic duct ending with the urogenital pore; s: sinusoids; bv: blood vessel; h: hepatocytes; white arrow: pectoral fin from the eyed-side showing the black colouration of the interradial membrane, characteristic of *S. senegalensis*; g: gills; ga: gills arch; m: mouth; pf: pelvic fins; c: capillary; cc: chloride cell; mc: mucous cell; pc: pillar cell; pl: primary lamella; sl: secondary lamellae; C: cortex; sl: seminiferous lobules; M: medulla; spz: spermatozoa present in the lumen of the lobules; black asterisks: different phases of atretic oocytes.

2.3. The use of *Solea* spp. in ecotoxicology

Based on the species characteristics (e.g. wide geographic distribution, high trophic position, benthic behaviour), *Solea* spp. shows great potential as sentinel species for the assessment of the general health status of the coastal and estuarine ecosystems in the SE Bay of Biscay, along the Iberian coast and in the Mediterranean Sea. Indeed, it has been introduced in previous field studies in the Bay of Biscay (Claireaux et al., 2004; Gilliers et al., 2006; Cuevas et al., 2015a, 2015b; Chapter 1), in Portugal (Vinagre et al., 2006; Fonseca et al., 2011a, 2011b; Gonçalves et al., 2013, 2014) and in the Mediterranean Sea (Jebali et al., 2013; Siscar et al., 2013, 2015; Oliva et al., 2010, 2012a, 2012b, 2013, 2014; Solé et al., 2013, 2016). In parallel, its sensitivity to contaminants has been studied in laboratory conditions upon exposure to waterborne pollutants (Solé et al., 2008; Oliva et al., 2009; López-Galindo et al., 2010a, 2010b; Díaz-Garduño et al., 2018), natural sediments (Riba et al., 2004; Costa et al., 2008, 2009a, 2009b; Jiménez-Tenorio et al., 2008; Ribecco et al., 2012; Ghribi et al., 2019), contaminated sediments (Salamanca et al., 2008; Martins et al., 2015) or via direct injections of chemicals (Costa et al., 2010b; Kalman et al., 2010). However, most of these experimental studies are focused on early life stages whereas studies using older juveniles or adults are rare. Alas, juveniles comprise an interesting age-class for biomonitoring programmes, as they are regularly found in estuarine and coastal areas where they inhabit for 2 to 3 years (Koutsikopoulos et al. 1989). Thus, juveniles have the potential to be representative of specific geographic areas where they spend enough time to become good indicators of ecosystem health disturbance (Gilliers et al., 2006). As a matter of fact, field studies using sole as sentinel species of estuarine and coastal ecosystems often use 2-3 yr old juveniles and adults (Claireaux et al., 2004; Oliva et al., 2010, 2012a, 2012b, 2013, 2014; Gonçalves et al., 2013, 2014; Jebali et al., 2013; Sànchez-Nogué et al., 2013; Siscar et al., 2013, 2015; Solé et al., 2013; Cuevas et al., 2015a, 2015b; Chapter 1). Therefore, laboratory studies on the biological responses of sole juveniles to environmental stressors (e.g. exposure to pollutants) are necessary to properly design sampling and interpret data in biomonitoring programmes.

3. Biological responses to pollutants

Whilst chemical data inform on the presence of contaminants in the different compartments of the environment (water column, sediment, biota), biological responses provides data for assessing the effects of pollutants on the biota (Bayne, 1989; Peakall and Walker, 1994; Cajaraville et al., 2000; Lyons et al., 2010; Hylland et al., 2017a, 2017b). Biological responses need to be interpreted upon the integration of various levels of biological complexity including (a) alterations at molecular, biochemical, cellular and histopathological level, (b) disease prevalence, (c) growth and reproduction impairment, and (d) survival (OSPAR, 1998; Broeg et al., 2005; Law et al., 2010). Most commonly investigated target organs in fish are liver, gills, brain and gonads, as their cells are known to react to a direct or indirect exposure to a variety of contaminants.

3.1. The choice of target organs

Liver is a major target organ in ecotoxicology because it is involved in xenobiotic metabolism, storage and detoxification (Health, 1995; Hinton et al., 2001; van der Oost et al., 2003; Au, 2004). In fish, liver is not in direct contact with the chemicals found in the milieu but it is continuously exposed to them or their derivatives through blood and plays an important role in xenobiotic accumulation and detoxification (Yancheva et al., 2015; Salamat and Zarie, 2016). Detoxification mechanisms include the biotransformation of endogenous and xenobiotic compounds into metabolites (phase I and phase II reactions) that can be eliminated from the cell or sequestered to avoid adverse toxic effects (Newman, 2015). The liver is also essential for the production of vitellogenin, an oestrogen-induced protein essential for female gamete development, which has been previously detected in males exposed to pollutants such as endocrine disruptors (Gonçalves et al., 2014). Liver samples are commonly used for chemical analysis to demonstrate the bioavailability and bioaccumulation of toxic compounds (Hinton and Laurén, 1990; van der Oost et al., 2003; Yancheva et al., 2015).

Brain, which is sensitive to a wide variety of contaminants (Soengas and Aldegunde, 2002), has attracted great interest in ecotoxicology due to its central role? in neural activities (e.g. swimming behaviour), crucial for fish survival (Birnie-Gauvin et al., 2017). This organ is also involved in the neuroendocrine control of reproduction through the synthesis of neurosteroids (Diotel et al., 2011). Neurotoxicity effects include neurodegenerative damage, altered sensory capacities, brain necrotic lesions and behavioural changes (e.g. muscular twitching, paralysis, altered swimming capacity). Moreover, the brain is also considered a target organ for assessing oxidative stress (Vieira et al., 2018).

Gills are also a major target organ for ecotoxicological studies, as it is continuously in direct contact with the milieu (Bernet et al., 1999; Au, 2004; Alvarado et al., 2006, 2007; Costa et al., 2009b; Lujić et al., 2013; Yancheva et al., 2015). Thus, gills are the first contact with waterborne pollutants and the main site for their uptake (Alvarado et al., 2006, 2007; Costa et al., 2009b; Lujić et al., 2013), and the concentrations of chemicals in gills seemingly reflect their concentration in the surrounding water (Yancheva et al., 2015). Based on the same principle of direct contact, gill histopathology can provide earliest evidence of biological effects exerted by pollutants (Costa et al., 2009b; Kalman et al., 2010).

In addition to their role in gamete development and reproductive functions, gonads have also attracted great interest as target organ for toxicology studies to detect the effects of xenobiotic compounds and in particular endocrine disrupting chemicals (Stentiford et al., 2003; Ortiz-Zarragoitia and Cajaraville, 2010; Bizarro et al., 2014; Dias et al., 2014; Gonçalves et al., 2014). Indeed, exposure to chemicals may impair reproductive function (Kime, 1995; Blazer, 2002) and generate specific biological effects such as e.g. the presence of plasma vitellogenin in males (Sumpter and Jobling, 1995; Gonçalves et al., 2014), abnormal sex hormone levels (Solé et al., 2016), the occurrence of intersex (Minier

et al., 2000; Bateman et al., 2004; Stentiford and Feist, 2005; Bizarro et al., 2014; Feist et al., 2015) and oocyte atresia (Blazer, 2002; Reynolds et al., 2003; Ortiz-Zarragoitia and Cajaraville, 2010).

3.2. The biomarker approach

The biomarker approach was developed for biomonitoring programmes to complement chemical analysis and to achieve an integrative assessment of the general health status of aquatic ecosystem (JAMP, 2003; Davies and Vethaak, 2012; OSPAR Commission, 2013). From molecular to community levels, biomarkers are early-warning biological responses to chemical(s) that can be classified into two categories: exposure and effect biomarkers (McCarthy and Shugart, 1990; Peakall and Walker, 1994; UNEP/RAMOGE, 1999; Lam, 2009). Exposure biomarkers focus on the assessment of specific reactions such as biotransformation and detoxification processes (Broeg et al., 2005) and are assessed at molecular and cellular levels. Effect biomarkers indicate the magnitude of the biological response to exposure to contaminants. They integrate the toxicity of various contaminants and thus are used as signs of general environmental deterioration. Different biomarkers from molecular to community levels are applied in a suite as they differ in specificity, sensitivity, response-time and ecological relevance (Figure 6; van der Oost et al., 2003). The use of batteries of biomarkers is a key strategy for biomonitoring programmes aimed at understanding the biological effects of exposure to contaminants in an integrative way (UNEP/RAMOGE, 1999; Cajaraville et al., 2000; Beliaeff and Burgeot 2002; Broeg et al., 2005; Kopecka et al., 2006; Viarengo et al., 2007).

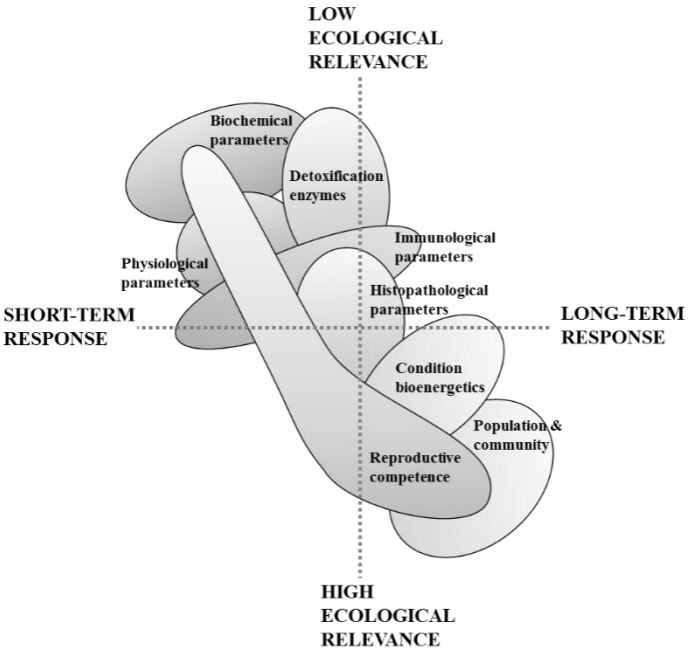

Figure 6. Relationships between ecological relevance and time-scales of pollutant-induced biomarker responses. Modified by van der Oost et al. (2003) from Adams et al. (1989).

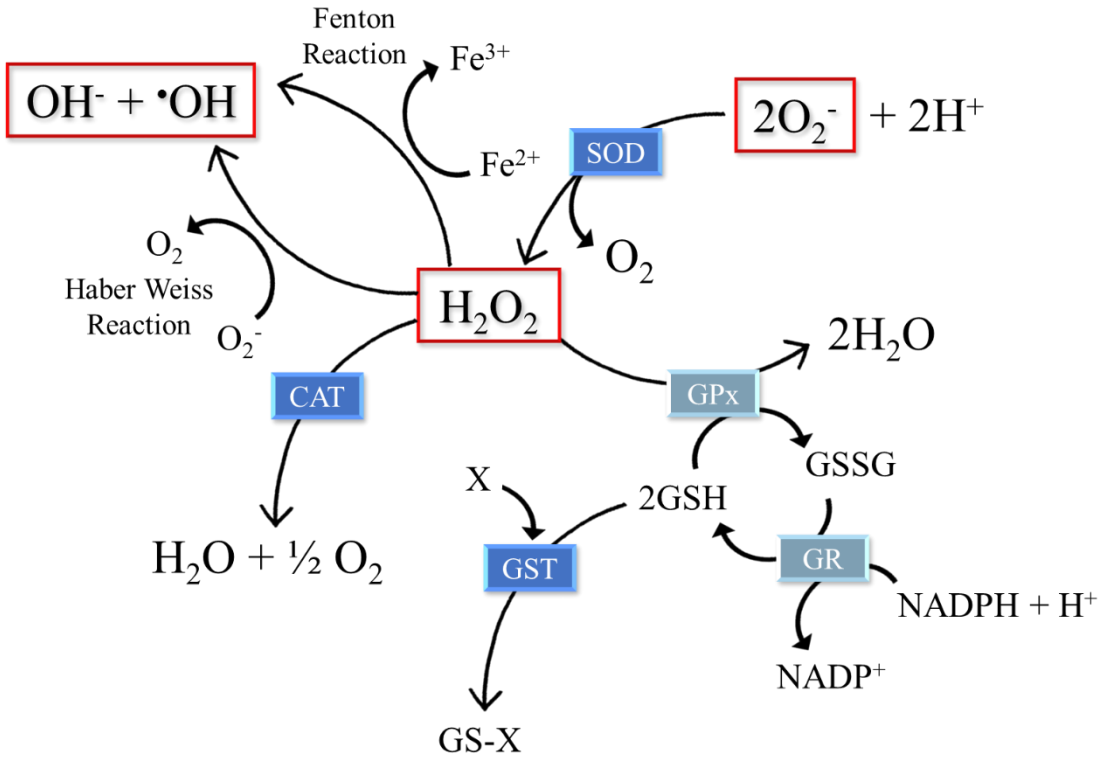

Figure 7. Main biochemical reactions involved in ROS production (Haber Weiss and Fenton reactions) and in cellular antioxidant defenses. Scheme adapted from Regoli and Giuliani, 2014.

Oxidative stress. In fish, like in most marine organisms, the antioxidant system is responsible for the maintenance of the prooxidant-antioxidant balance (Regoli and Giuliani, 2014). Exposure to environmental stressors can perturb this equilibrium and generate oxidative damage with enhanced production of reactive oxygen species (ROS), disturbed antioxidant defences and altered xenobiotic metabolism (Sies et al., 1991; Livingstone, 2001; Regoli and Giuliani, 2014).

The production of ROS, stimulated by natural and anthropogenic compounds, is linked to several cellular pathways of aerobic metabolism. The two main biochemical reactions involved in ROS formation are the Haber Weiss and Fenton reactions (Figure 7). Antioxidant defences to prevent the effects of oxyradicals are based on the activity of scavengers and antioxidant enzymes working in a complex network (Regoli and Giuliani, 2014). Scavengers interact directly with ROS for their neutralisation and can react with different types of ROS. In comparison, antioxidant enzymes are specific to their substrates. Amongst the different pathways involved in antioxidant defences, the superoxide dismutase (SOD) is responsible for the scavenging of superoxide anion radicals (Figure 7). This enzyme is present in different cellular components such as the cytoplasm, mitochondria and peroxisomes. The reaction catalysed by SOD produces hydrogen peroxide (H_2O_2) which implies the activity of H_2O_2 reducing enzymes, namely the catalase (CAT) and the glutathione peroxidases (GPx). CAT is mainly found in peroxisomes and is also involved in the detoxification of phenols and alcohols as coupled

reactions to the reduction of H_2O_2. The role of CAT is essential in antioxidant defences as it permits to reduce the availability of H_2O_2 for the production of highly reactive initiators of membrane lipid peroxidation by the Fenton reaction (Figure 7; Regoli et al., 2002). H_2O_2 is also reduced by the glutathione peroxidase (GPx) in parallel with the oxidation of reduced glutathione (GSH) into the oxidized glutathione (GSSG). Xenobiotic and damaged endogenous compounds can be eliminated via their conjugation with GSH into the metabolite GS-X (Figure 7). This reaction is one of the many conjugation reactions catalysed by the glutathione-*S*-transferase (GST; Regoli and Principato, 1995).

Changes in the activity of antioxidant enzymes are well-known biological responses to environmental stressors in animals (Jee and Kang, 2005; Valavanidis et al., 2006; Salamanca et al., 2008; Oliva et al., 2010, 2012b; Siscar et al., 2015; Kroon et al., 2017); however, their interpretation can be controversial (van der Oost et al., 2003). In sole, the activity of these enzymes in response to pollutants can be either induced (Jiménez-Tenorio et al., 2008; Salamanca et al., 2008; López-Galindo et al., 2010a; Fonseca et al., 2011a) or inhibited (López-Galindo et al., 2010b; Oliva et al., 2012b; Gonçalves et al., 2013; Díaz-Garduño et al., 2018), or both (Wu et al., 2006; Gravato et al., 2009; Mani et al., 2014).

Neurotoxicity. The potential neurotoxic effect of contaminants can be assessed by changes in acetylcholinesterase (AChE) activity, an enzyme involved in neural transmission (Davies and Vethaak, 2012; Oliva et al., 2012a; Burgeot et al., 2017). In vertebrates such as fish, the acetylcholine (ACh) is released in the nerve synapses where it acts as an excitatory transmitter (Fulton and Key, 2001). AChE catalyses the hydrolysis of ACh to regulate the presence of the neurotransmitter in the synaptic gap. Enzymatic inhibition results in the accumulation of ACh, which generates a continuous stimulation of receptors located in the post-synaptic membrane. Subsequent abnormal nervous functions include rapid muscular twitching and paralysis. Initially, AChE inhibition was mainly used as neurotoxic biomarker of organophosphate and carbamate pesticides (WHO 1986a, 1986b; Grue et al., 1997; Heath et al., 1997; Davies and Vethaak, 2012). AChE sensitivity has been demonstrated for a wide range of chemicals and the enzyme is now used as biomarker of general stress in aquatic organisms, including sole (López-Galindo et al., 2010a, 2010b; Oliva et al., 2012a; Solé et al., 2012; Jebali et al., 2013; Siscar et al., 2013).

Lysosomal responses. As part of the endo-lysosomal system (Figure 8), lysosomes are responsible for the recycling of damaged cellular components and the accumulation and degradation of exogenous substances that reached the intracellular compartment (De Duve, 1983; Lin and Steichen, 1994; Hu et al., 2015). They contain a high amount of hydrolases whom activity is pH-dependent. The acidic pH of the lysosome ensures their activation in the vesicle and the neutral pH of the cytosol keeps them inactive, preventing from autodegradation. Lysosomal pH is maintained via the action of a proton pump in the lysosomal membrane (Figure 8). The description of the endo-lysosomal system and

lysosomal dysfunctions in human medicine has attracted great interest for toxicological studies to identify potential cellular effects of pollutants. Amongst the material reaching lysosomes, a wide range of chemicals such as metallic and organic contaminants can be found sequestrated in the vesicle where they are detoxified (Moore, 2004; ICES, 2011; Davies and Vethaak, 2012). Pollutants load in lysosomes may exceed the vesicle's storage capacity and alter its structure (e.g. lysosomal enlargement) and the integrity of its membrane (membrane destabilisation; Einsporn et al, 2005).

Lysosomal changes for environmental research were first studied and applied in marine molluscs (Moore, 1976, 1982, 1985; Regoli, 1992; Lin and Steichen, 1994; Cajaraville et al., 1995; Marigómez et al., 1996) and later in fish species (Köhler, 1991, 2002; Köhler et al., 1992; Broeg et al., 1999; Diamant et al., 1999). They are considered sublethal to lethal responses to environmental factors; they are not specific to the action of pollutants but instead are representative of the subsequent adverse health effects of exposure to contaminants or other stress factors (Davies and Vethaak, 2012). Changes in lysosomal structure and membrane integrity are considered biomarkers of general stress (Regoli, 1992; Broeg et al., 1999, 2002; UNEP/RAMOGE 1999; Köhler et al. 2002; JAMP, 2003; Moore, 2004; Moore et al., 2008, 2013; ICES, 2006; Davies and Vethaak, 2012). In laboratory studies, lysosomal changes have been reported in response to a variety of contaminants such as metals (Roméo et al., 2000; Alvarado et al., 2005; Giambérini and Cajaraville 2005; Marigómez et al., 2005a; Izagirre et al., 2014; Brooks et al., 2015; Benito et al., 2017) and PAHs (Marigómez and Bay-bay-Villacorta, 2003; Marigómez et al., 2005a; Blanco-Rayón et al., 2018). Their application in biomonitoring programmes

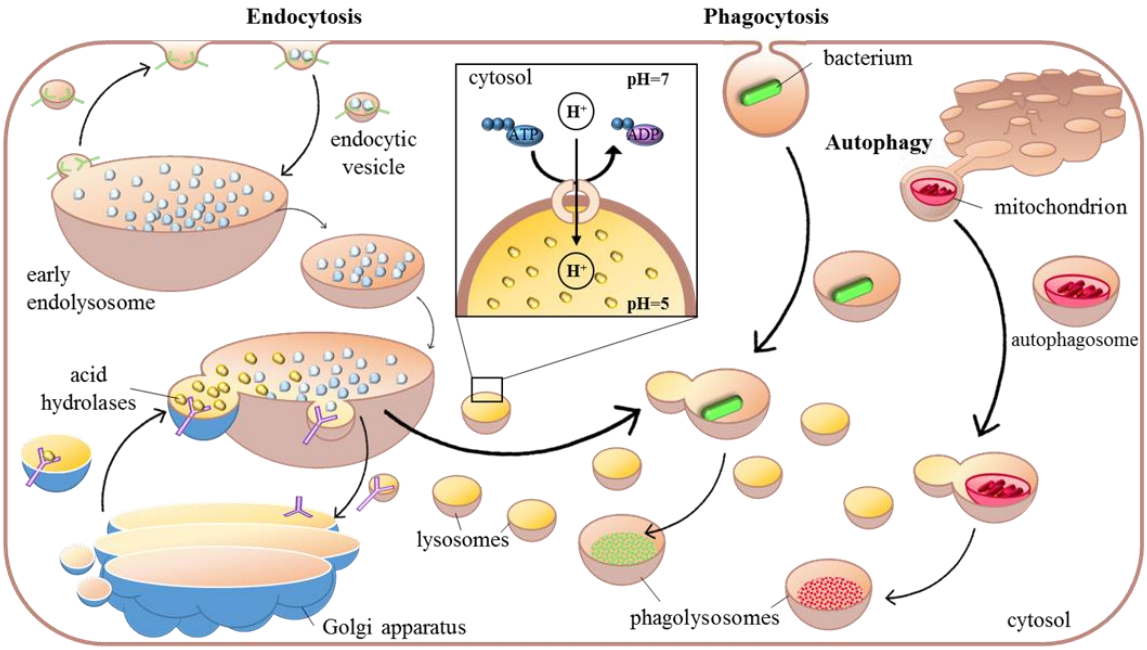

Figure 8. Schematic representation of the endo-lysosomal system showing different entry pathways of exogenous substances into lysosomes. Scheme adapted from Cooper, 2000.

allowed for the assessment of the general health status of the marine ecosystem, using bivalves (Cajaraville et al., 2000; Domouhtsidou and Dimitriadis 2001; Koukouzika and Dimitriadis, 2005; Marigómez et al., 2006; Da Ros et al., 2007; Viarengo et al., 2007; Diaz de Cerio et al., 2018; Benito et al., 2019), fish (Bilbao et al. 2006; Schiedek et al. 2006; Zorita 2008) and in particular flatfish species (Kohler 1991; Broeg et al. 2002, 2005; Alvarado et al. 2005; Einsporn et al., 2005; Baršienė et al., 2006; Burgeot et al., 2017).

Lysosomal membrane stability. Among lysosomal alterations, cellular effects of exposure to environmental factors include the destabilisation of the vesicle's membrane (UNEP/RAMOGE, 1999; Köhler et al., 2002; Davies and Vethaak, 2012). Damage of the lysosomal membrane may affect the permeability of the vesicle and let hydrolases reach the cytosol (Lin and Steichen, 1994). Although the enzymes activity is pH-dependent and should be inactive in the cytoplasm, hydrolases leakage from several lysosomes may affect the cytosolic pH and generate degenerative processes with cellular autolysis and potential cell death (Bayne et al., 1976; Einsporn et al., 2005).

The lysosomal membrane integrity is a cellular biomarker of sublethal effects of environmental stressors and is used to assess changes in the general health status of the aquatic environment (Cajaraville et al., 1995; Marigómez and Bay-bay-Villacorta, 2003; Moore, 2004; Moore et al., 2007, 2013; Izagirre et al., 2008; Davies and Vethaak, 2012; ICES, 2015). It can be assessed based on the lysosomal membrane stability test, which was recognised by the UN Environment Programme (UNEP) for its application in the Mediterranean Pollution programme (MED POL) and included in marine monitoring frameworks (Baršienė et al., 2006; OSPAR Commission, 2010; Davies and Vethaak, 2012; HELCOM, 2012; UNEP/MAP, 2014). The test is based on the demonstration of the latent activity of lysosomal hydrolases found in fish hepatocytes such as the acid phosphatase (AcP) (Figure 9A; UNEP/RAMOGE, 1999; ICES, 2004). The integrity of the membrane is tested by application of different intervals of acid labilisation. Membrane destabilisation generates an input of the enzyme's substrate into the vesicle, which is detected by an increased staining of the lysosome. The destabilisation time leading to the maximum staining intensity is defined as the labilisation period (LP, in min). Thus, longer LP shows higher lysosomal membrane integrity whilst lower values demonstrate an increased lysosomal permeability (Köhler et al., 2002). Based on this technique, sublethal effects of general stressors are detected by decreased LP values. As a general observation, healthy animals tend to show a LP>20 min and severely stressed individuals would show values <10 min (Viarengo et al., 2000, 2007; Broeg et al., 2005; Moore et al., 2006). Lysosomal membrane destabilisation has been reported in fish liver in response to environmental stressors (Broeg et al., 1999, 2002; Köhler et al., 2002; Baršienė et al., 2006; Zorita et al., 2008; Burgeot et al., 2017). However, changes in lysosomal membrane stability are not always so clear, in particular in case of low contaminant concentrations where lysosomes may appear more stable (Marigómez et al., 2005b; Izagirre and Marigómez, 2009). Thus, the assessment of the lysosomal membrane stability in

combination with other lysosomal biomarkers is essential for a correct interpretation of contaminants effects.

Lysosomal Structural changes.
Possible lysosomal responses to exposure to stress factors also include changes in lysosomal structure such as changes in size and number. Lysosomal size varies with the nature and quantity of material (e.g. pollutants) reaching the organelle where it is either metabolised and eliminated or accumulated (Köhler et al., 2002).

Alterations in lysosomal structure can be assessed by measurement of stereological parameters after demonstration of the latent activity of the lysosomal enzyme β-glucuronidase (Figure 9B). Lysosomal enlargement for instance, may be characterised by an increase in lysosomal volume density (Vv_L), a decrease in lysosomal surface-to-volume ratio (S/V_L, value inverse to lysosomal size) and a decrease in lysosomal numerical density (Nv_L). Lysosomal enlargement has been recorded in fish hepatocytes as cellular responses to environmental stressors (Köhler, 2004; Alvarado et al., 2005). Reference values for lysosomal parameters are available for bivalves (Davies and Vethaak, 2012); however, less data are available from flatfish species (Köhler, 2004; Alvarado et al., 2005).

Changes in lysosomal content.
Environmental stressors may also alter the lysosomal content notably with increased accumulation of neutral lipids (Viarengo et al., 2007). The storage of neutral lipids such as triglycerides, steryl esters, and retinyl esters in intracellular

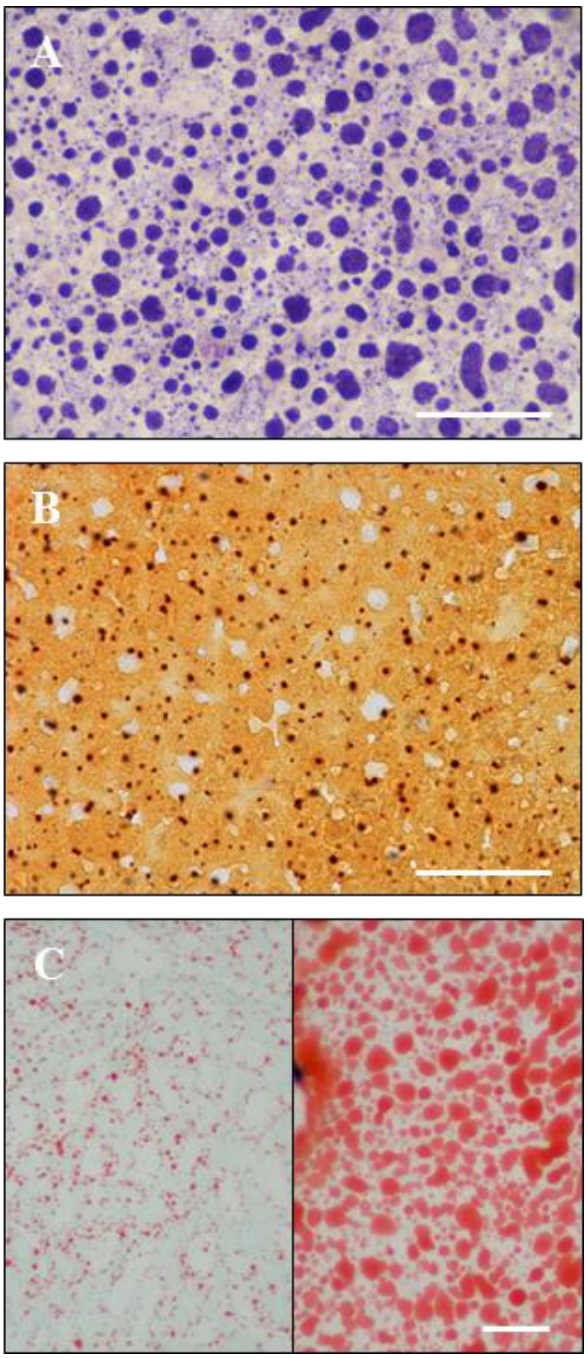

Figure 9. Micrographs of hepatic histochemical sections of *S. senegalensis* showing the demonstration of lysosomal enzymes activities, the acid phosphatase (A) and β-glucuronidase (B), and the accumulation of neutral lipid droplets (C). White scale: 50 µm.

droplets is essential for energy homeostasis and lipid metabolism (Welte, 2015). These lipid droplets are also involved in the sequestration of toxic compounds. Intracellular accumulation of neutral lipids in fish hepatocytes can be quantified after staining of liver sections with Oil Red O (ORO; Culling, 1974; Figure 9C) and is expressed as volume density of neutral lipids (Vv_{NL}). Increased neutral lipid accumulation has been recorded in fish species after exposure to contaminants (Viarengo et al., 2007) and was recognised as an early indicator of liver injury (Köhler et al., 2002; Köhler, 2004).

Histopathology. Biological responses to exposure to environmental stressors identified by cellular biomarkers (oxidative stress, neurotoxicity, altered endo-lysosomal system) may progress to autophagy and apoptosis (Moore et al., 2006; Chiarelli et al., 2016) and potentially generate tissue-level alterations (Köhler et al., 2002, 2004). Histopathological lesions are considered indicators of biological effects of sub-lethal and chronic exposures to environmental stressors (Bernet et al., 1999; Reynolds et al., 2003; van der Oost et al., 2003; Stentiford et al., 2003; Stentiford and Feist, 2005; Costa et al., 2009b, 2010a, 2010b; Gonçalves et al., 2013; Feist et al., 2015). They are considered non-specific biological responses as they may originate from exposure to various environmental stressors such as mixtures of contaminants (Yancheva et al., 2015). Histopathological approaches are advantageous for ecotoxicology studies because they allow for the assessment of medium-term responses that can be extrapolated to community and ecosystem levels (Hinton and Lauren, 1990; Au, 2004; Lang et al., 2006). Fish histopathology has been applied as an integrative method for environmental health assessment in monitoring programmes (OSPAR QSR 2000; HELCOM, 2002; Stentiford et al., 2003, 2009; Lang et al., 2006; Salamat and Zarie, 2016).

In particular, liver histopathology is recognised as a sensitive tool for the assessment of the biological effects of environmental stressors (Bucke et al., 1996; Bernet et al., 1999; Feist et al., 2004; Costa et al., 2009b, 2013; Fricke et al., 2012), and has been implemented in the OSPAR Joint Assessment and Monitoring Programme (JAMP) together with quality guidelines pursuing standardised procedures (ICES, 1997; BEQUALM, 2001; Feist et al., 2004). Liver histopathology in sole has been investigated in laboratory (Arellano et al., 1999; Salamanca et al., 2008; Costa et al., 2009b, 2011, 2013; Oliva et al., 2009) and field studies (Gonçalves et al., 2013; Oliva et al., 2013; Cuevas et al., 2015a, 2015b; Chapter 1).

As previously mentioned, melanomacrophage centres may be identified throughout the parenchyma of healthy tissues. However, their frequency and staining intensity are also known to change with infectious diseases or chemical exposure (melanomacrophage centres accumulation: MMCs; Zorita and Cuevas, 2014). Along with lymphocytic infiltration, increased prevalence of MMCs is a common inflammatory response recorded as indicator of altered immune system (Agius and Roberts, 2003; Stentiford et al., 2003). They are often coupled with circulatory disturbances such as hyperaemia (Noreña-Barrose et al., 2004; Zorita and Cuevas, 2014) and haemorrhage (Costa et al., 2011). These lesions are early tissue-level indicators of environmental stressors and are

considered of minor severity, as they are reversible. In comparison, necrosis is an irrevocable degenerative alteration characterized by loss of cellular structure and functions (Oliveira Ribeiro et al., 2005; Costa et al., 2013). Another example of regressive change is the identification of hepatocellular and nuclear pleomorphism (HNP), often observed after exposure to carcinogenic compounds (Köhler, 1990). One of the liver's main functions is the storage of lipids as source of energy. The degree of hepatocytes fat content varies with food availability and gamete development but excessive fat vacuolation has also been observed in response to exposure to xenobiotic compounds (Costa et al., 2009b, 2011). Other progressive changes include the hydropic vacuolation of epithelial cells of bile duct and concentric periductal fibrosis which are commonly related to parasitic infection. Cases of neoplastic lesions have been described in response to exposure to contaminants in fish and in particular in flatfish species (Köhler and Pluta, 1995; Vethaak et al., 1996; WGBEC, 2002; Lang et al., 2006; Stentiford et al., 2009, 2010; Zorita and Cuevas, 2014; Feist et al., 2015; Chapter 1).

Gill histopathology allows for the assessment of the biological effects of recent stressors (Costa et al., 2009b; Kalman et al., 2010). This approach is not contaminant-specific; instead, it is representative of the general health status of individuals and of environmental quality (Arellano et al., 1999; Au, 2004). Gill histopathological lesions are indicative of potential physiological effects such as altered osmoregulation and respiration, which can have severe consequences on survival (Sensini et al., 2008).

Direct exposure to environmental stressors may damage the structure of gills and alter its physiological functions (Au et al., 2004; Lowe et al., 2015). For instance, suspended sediments, among other stressors, are known to affect the length of gill lamellae and alter the gill epithelium (Smart, 1976; Mallat, 1985; Hinton and Laurén, 1990; Arellano et al., 2004; Hess et al., 2017). This can be translated by the histological observation of regressive changes (epithelial lifting and desquamation). One strategy to compensate epithelial damage and to reduce the effect of toxic compounds is to increase the thickness of gill epithelium by proliferation of lamellar cells. This histological lesion is called epithelial hyperplasia (Roberts, 2001) and can progress into lamellar fusion, both lesions impeding gas exchanges (Skidmore and Tovel, 1972). Other common progressive changes include hypertrophy of pavement cells and chloride cells. Severe exposure to environmental stressors can also damage the structure of pillar cells, which would alter the lamellar blood flow and generate circulatory disturbances such as blood congestion, aneurysm and haemorrhage (Martinez et al., 2004; Camargo and Martinez, 2007). They are considered more severe than epithelial alterations in the sense that recovery is more difficult. Gills histopathological alterations have been reported in response to a variety of contaminants including contaminated sediments (Stentiford et al., 2003; Jímenez-Tenorio et al., 2008; Costa et al., 2009b, 2010a; Oliva et al., 2013; Martins et al., 2016; Chapter 1), waterborne metals (Arellano et al., 1999; Martinez et al., 2004; Oliva et al., 2009; Costa et al., 2013) and PAHs and pesticides (van der Oost et al., 2003; Noreña-Barroso et al., 2004; Camargo and Martinez, 2007; Salamanca et al., 2008; Lopez-Galindo et al., 2010; Martins et al., 2016; Salamat and Zarie, 2016).

Gonad histo(patho)logy gives essential data on the reproduction status of a population and on its preservation for future generations (Blazer, 2002; Solé et al., 2016). The identification of histopathological lesions can also contribute to identify the effects of endocrine disrupting chemicals. These lesions include the occurrence of intersex based on the detection of ovotestis or testis-ova (Minier et al., 2000; Bateman et al., 2004; Stentiford and Feist, 2005; Bizarro et al., 2014; Feist et al., 2015) and oocyte atresia (Blazer, 2002; Reynolds et al., 2003; Ortiz-Zarragoitia and Cajaraville, 2010). Oocyte atresia can occur naturally as part of the gamete development but it has also been identified in response to environmental stressors (Witthames et al., 1995; Reynolds et al., 2003; Chapter 1).

Although histopathological approaches provide essential data on the general health status of individuals, one major limitation remains on the lack of quantitative methods. The acquisition of numerical data aims to help evaluating the biological significance of histopathological lesions and to enhance studies comparability. The calculation of lesion prevalence gives a first numerical data that informs on the lesion commonness or rarity in a group of individuals but lacks to represent the differences in lesion severity. Histopathological indices can be calculated based on semi-quantitative methods that takes into account the severity of the lesion identified (weight) and the level of dissemination in the organ (score) (Bernet et al., 1999; Lang et al., 2006; Van Dyk et al., 2007; Triebskorn et al., 2008; Costa et al., 2009b). They are suitable for the assessment of biological effects of exposure to contaminants and have been applied in sole and other flatfish species (Costa et al., 2009b, 2011; Gonçalves et al., 2013; Cuevas et al., 2015).

Similarly, biological responses assessed through the application of a battery of biomarkers can be integrated into indices to obtain a numerical representation of changes in ecosystem health. Amongst the different integrative indices proposed (Chèvre et al., 2003; Broeg et al., 2005; Dagnino et al., 2007; Izagirre and Marigómez, 2009; Marigómez et al., 2013), the Integrated Biomarker Response (IBR) index is calculated based on the area defined by biomarkers when arranged into a star plot (Beliaeff and Burgeot, 2002; Sanchez et al., 2013; Devin et al., 2014). The method is considered to be a sensitive tool for the assessment of changes in general health status of the aquatic biota for biomonitoring programmes (Broeg and Lehtonen 2006; Brooks et al., 2011; Cravo et al., 2012; Serafim et al., 2012; Marigómez et al., 2013; Rementeria et al., 2016).

4. Ecotoxicological experimentation with fish

Ecotoxicology assays are developed to understand how contaminants may affect organisms, populations and communities. Acute toxicity tests are usually the first step to estimate potential adverse effects of a new contaminant. They are based on the calculation of quantitative indicators such as the LC50 (median lethal concentration; Erhirhie et al., 2018). Nevertheless, the environmental relevance of these tests for European ecosystems has been questioned as their application is recommended for certain fish species that are not present in European waters (Braunbeck et al., 2005). Instead, fish embryo tests using natural population are suggested as substitute of acute toxicity tests (Braunbeck et al.,

2005; Lammer et al., 2009; Halder et al., 2010; Belanger et al., 2013). They are considered reproducible, time and cost-effective and may include different toxicological endpoints (Wedekind et al., 2007). As embryo toxicity tests are not suitable for cases of chronic contamination, laboratory assays including larvae, juveniles and adults are also necessary.

In the attempt to resemble the complexity of the environment, ecotoxicology assays using older individuals must take into account a variety of contaminants at different concentrations, and a variety of exposure patterns and exposure time (Table 1). Typically, chemical toxicity assessment includes exposure to different concentrations of the studied contaminant. High concentrations of single contaminant are applied to induce rapid and acute biological effects whilst the use of environmentally relevant concentrations aims to establish the association between levels of chemicals encountered in the field and alterations in the health status recorded in the biota. Similarly, the exposure pattern may vary between toxicity assays (Table 1); it must be relevant to the environmental pathways of the studied contaminant. In the case of selenium for instance, environmental toxicity is based on dietary exposure and progresses through the food chain (Chapman, 1999). Thus, although standard ecotoxicology assays using waterborne contaminant were essential to demonstrate the acute toxicity of selenium, it was also crucial to include assays using dietary exposure to selenium (Janz et al., 2010). Dietary exposure is also commonly used to assess potential bioaccumulation of contaminants and to help understanding mechanisms involved in detoxification processes (Le Croizier et al., 2019). In the environment, contaminants rarely occur isolated; instead, they are found as a complex mixture. Toxicity assays using chemical mixtures are considered environmentally realistic and assess antagonistic or synergistic effects of contaminants. Costa et al. (2010) for instance, demonstrated that the combination of Cd and B[a]P upon hepatic injection in sole induced different toxicity than isolated contaminants. In the environment, mixtures of contaminants are also found in the sediment. The complexity of this milieu is related to its dual capacity to trap and release chemical compounds to the water column (Eggleton and Thomas, 2004). This changing bioavailability of contaminants depends on environmental conditions (pH, salinity, temperature) and sediment characteristics (granulometry, redox potential). Sediment toxicity is also influenced by the different relative concentration, speciation and mobility of each contaminant (Chapman, 1990). Toxicity tests assessing sediment contamination include exposure to naturally contaminated sediments and spiked sediments (Table 1; Hallare et al., 2011). Most ecotoxicology studies on sediment contamination focus on toxicity for the benthic community including crustaceans (e.g. amphipods), polychaete, molluscs and fish, in particular flatfish (Table 1). This diversity of ecotoxicological assays (wide range of contaminants, various exposure pathways, mixture of contaminants, among others) is essential to support field studies surveying the health status of the aquatic ecosystem prone to a complex and fluctuating contamination.

Table 1. The place of *Solea* spp. in fish ecotoxicology. Different exposure patterns, contaminants and exposure time in recent toxicological experiments. WE: Waterborne Exposure; SE: Sediment Exposure; INJ: Injection; FS: Field Study; DE: Dietary Exposure; T: Temperature; NA: Not Applied.

Reference	Exposure pattern	Contaminants	Exposure time	Fish sp.	Biological endpoints
Arellano et al., 1999	WE	Metals (Cu)	7 d	*S. senegalensis*	Liver and gill histopathology
Briaudeau et al., 2019	FS	Natural sediments	7 yr	*Solea* spp.	Multi-organ histopathology
Claireaux and Davoodi, 2002	WE	PAHs (oil)	5 d	*S. solea*	Impaired cardio-respiratory responses
Claireaux et al., 2004	WE	PAHs (oil)	5 d	*S. solea*	Biochemistry and histopathology
Costa et al., 2008	SE	Natural sediments	28 d	*S. senegalensis*	Genotoxicity
Costa et al., 2009a	SE	Natural sediments	28 d	*S. senegalensis*	Biochemistry
Costa et al., 2009b	SE	Natural sediments	28 d	*S. senegalensis*	Liver and gill histopathology
Costa et al., 2010a	SE	Natural sediments	28 d	*S. senegalensis*	Gills and kidney histopathology
Costa et al., 2010b	INJ	Multiple	NA	*S. senegalensis*	Proteomic and liver histopathology
Costa et al., 2011	SE	Natural sediments	28 d	*S. senegalensis*	Liver histopathology
Costa et al., 2013	WE	Metals (Cd)	28 d	*S. senegalensis*	Multi-organ histopathology
Cuevas et al., 2015a	FS	NA	1 yr	*S. solea*	Multi-organ histopathology
Cuevas et al., 2015b	FS	Natural sediments	2 yr	*S. solea*	Multi-organ histopathology
Díaz-Garduño et al., 2018	WE	Urban effluent	7 d	*S. senegalensis*	Biochemistry
Fonseca et al., 2011a	FS	Natural sediments	<1 yr	*S. senegalensis*	Biochemistry
Fonseca et al., 2011b	FS	Env. Param.	<1 yr	*S. senegalensis*	Biochemistry
Ghribi et al., 2019	SE	Natural sediments	28 d	*S. senegalensis*	Biochemistry and histopathology
Gilliers et al., 2006	FS	Oil spill	1 m	*S. solea*	Growth indicators and biochemistry
Gonçalves et al., 2013	FS	Multiple	<1 yr	*S. senegalensis*	Biochemistry and histopathology
Gonçalves et al., 2014	FS	Organic toxicants	<1 yr	*S. senegalensis*	Biochemistry and gonad histopathology
González-Mira et al., 2016	INJ	Pharmaceutical drugs	NA	*S. senegalensis*	Biochemistry
Hampel et al., 2008	SE	Spiked sediments	30 d	*S. senegalensis*	Histopathology and larvae survival
Jebali et al., 2013	FS	Multiple	1 m	*S. solea*	Biochemistry and histopathology

Reference	Exposure pattern	Contaminants	Exposure time	Fish sp.	Biological endpoints
Jiménez-Tenorio et al., 2008	SE	PAHs (oil)	42 d	*S. senegalensis*	Biochemistry and histopathology
Kalman et al., 2010	INJ	Metals (Cd)	NA	*S. senegalensis*	Histopathology
Le Croizier et al., 2018	WE	Metals (Cd)	2 m	*S. senegalensis*	Organotropism and metallothionein
Le Croizier et al., 2019	DE	Metals (Cd)	2 m	*S. senegalensis*	Metal partitioning
Lefrançois and Claireaux, 2003	O_2 and T	Env. Param.	48 h	*S. solea*	Heart rate and growth
López-Galindo et al., 2010a	WE	Organic antifoulant	7 d	*S. senegalensis*	Biochemistry and histopathology
López-Galindo et al., 2010b	WE	Organic antifoulant	15 d	*S. senegalensis*	Biochemistry
Martins et al., 2015	SE	Natural sediments	28 d	*S. senegalensis*	Histopathology
Oliva et al., 2009	WE	Metals (Cu)	96 hr	*S. senegalensis*	Histopathology
Oliva et al., 2010	FS	PAHs	4 yr	*S. senegalensis*	Biochemistry
Oliva, et al. 2012a	FS	Multiple	4 yr	*S. senegalensis*	Biochemistry
Oliva et al., 2012b	FS	Metals	4 yr	*S. senegalensis*	Biochemistry
Oliva et al., 2013	FS	Multiple	4 yr	*S. senegalensis*	Histopathology
Oliva et al., 2014	FS	Multiple	4 yr	*S. senegalensis*	Biochemistry
Riba et al., 2004	SE	Natural sediments	30 d	*S. senegalensis*	Metallothionein
Ribecco et al., 2012	SE	Natural sediments	96 hr	*S. solea*	Molecular biomarkers
Salamanca et al., 2008	WE	PAHs (oil)	21 d	*S. senegalensis*	Biochemistry and histopathology
Sànchez-Nogué et al., 2013	*in vitro*	Pesticides	30 min	*Solea* spp.	Biochemistry
Siscar et al., 2013	FS	Metals	1 yr	*Solea* spp.	Biochemistry
Siscar et al., 2014	T	Env. Param.	2 m	*S. senegalensis*	Metallothionein and metal bioaccumulation
Siscar et al., 2015	FS	NA	<1 yr	*Solea* spp.	Biochemistry
Solé et al., 2008	WE	PAHs (oil)	72 hr	*S. senegalensis*	Biochemistry
Solé et al., 2013	FS	Natural sediments	<1 yr	*Solea* spp.	Immunological test
Solé et al., 2016	FS	NA	<1 yr	*Solea* spp.	Biochemistry
Trisciani et al., 2011	FS	PAHs (oil)	<1 yr	*S. solea*	Molecular biomarkers and biochemistry
Vieira et al., 2018	FS	Env. Param.	2 yr	*S. solea*	Physiology and biochemistry
Wessel et al., 2010	WE	PAHs	28 d	*S. solea*	Biotransformation and biochemistry

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II. State of the Art, Hypothesis and Objectives

State of the Art

The anthropogenic pressure on the aquatic environment is undeniable and environmental protection has become a clear importance over the last 50 yr. Although legislative measures have been implemented, the improvement of environmental quality/health is a slow and complex process. Thus, even after the regulation of priority substances, contaminants are still present in the environment, in particular in the confined ecosystems of coastal and estuarine areas. Pollution monitoring programmes aim to detect and quantify contaminants present in the environment and to assess their potential effects on the surrounding biota. In relation with the increasing awareness on sediment contamination, a particular interest has been drawn towards the use of benthic organisms as sentinel species to assess the health status of coastal and estuarine ecosystems. The flatfish *Solea* spp. is abundant throughout the Iberian Peninsula and the Bay of Biscay. Its distribution, benthic behaviour, economic and ecological importance, and the knowledge acquired from previous studies on the species make the sole an attractive species for the assessment of estuarine and coastal ecosystems health status.

However, the sensitivity of the species to environmental contamination was mainly established in early life stages, based on biochemical and histopathological approaches. They correspond to larvae that migrate from deep spawning grounds to coastal areas during metamorphosis or to newly metamorphosed individuals that recently reached estuarine ecosystems. Thus, the use of sole larvae and fry for the assessment of estuarine ecosystem health may be limited by their age and short stay in the area. Instead, juveniles and young adults remain in estuaries and coastal areas during the first 2 to 3 years of life. Indeed, individuals of this age class are starting to be used in field studies to observe the health status of these ecosystems. In the laboratory, there is a growing number of ecotoxicology assays assessing the sensitivity of sole juveniles to environmental stressors (e.g. exposure to pollutants). This responsiveness can be assessed in an integrative way based on the application of a battery of biomarkers at different biological organization levels. Biochemical, cell and tissue-level biomarkers together with histopathology are considered early warning biological responses to exposure to chemicals.

In the attempt to approximate the complexity of environmental contamination, laboratory experiments consider a wide range of stressors such as contaminants, applied at different concentrations and in a variety of exposure patterns and exposure time. The use of native sediments for instance, allow for the assessment of their complex toxicity considering the mixture of contaminants, the relative concentration, speciation and mobility of each chemical and the influence of physicochemical parameters and sediment characteristics. However, the association between sediment contaminants present as a mixture and alterations in the health status recorded in the biota is intricate. Model contaminants used at different concentrations are also essential to identify toxicopathic effects of environmental stressors in controlled conditions.

Hypothesis

Solea spp. is responsive to environmentally realistic concentrations of pollutants, which can be quantified upon the application of an integrative battery of biomarkers and histopathology, and therefore it is suitable as sentinel species for the assessment of the biological effects of pollution in OSPAR Region IV biomonitoring programmes in the context of EU Marine Strategy Framework Directive.

Objectives

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

1- To assess and survey the ecosystem health status of an estuarine area experiencing recovery from a deteriorated situation, by implementation of a monitoring programme based on sediment chemistry and sole juveniles multi-organ histopathology.

2- To establish the association between sediment contamination and toxicopathic effects in sole juveniles upon a 28-day laboratory exposure based on chemical analysis and on the application of an integrative battery of biomarkers including biochemical, cellular and histopathological endpoints.

3- To confirm the suitability of a 7-day toxicity assay using a waterborne model metal (Cd) at different concentrations for the assessment of toxicopathic effects of environmental stressors in sole juveniles based on an integrative battery of biomarkers including biochemical, cellular and histopathological endpoints.

4- To confirm the suitability of a 7-day toxicity assay using a waterborne model organic contaminant (B[a]P) at different concentrations for the assessment of toxicopathic effects of environmental stressors in sole juveniles based on an integrative battery of biomarkers including biochemical, cellular and histopathological endpoints.

III. Results

Chapter I

Multi-annual survey of health status disturbance in the Bilbao estuary (Bay of Biscay) based on sediment chemistry and juvenile sole (*Solea* spp.) histopathology

Article:

Briaudeau T., Zorita I., Cuevas N., Franco J., Marigómez I., Izagirre U. (2019). Multi-annual survey of health status disturbance in the Bilbao estuary (Bay of Biscay) based on sediment chemistry and juvenile sole (*Solea* spp.) histopathology. Marine Pollution Bulletin, 145: 126-137.

Congress:

Briaudeau T., Zorita I., Cuevas N., Franco J., Lekube X., Marigómez I., Izagirre U. (2019). Bilbao estuary pollution monitoring programme: retrospective studies of the environmental health status using sole (*Solea* spp.) as sentinel species. Environmental Specimen Banks Conference 2019, Stockholm, Sweden, 3-5 June 2019, Platform Presentation (U. Izagirre).

Abstract

The Bilbao estuary (SE Bay of Biscay) is a recovering ecosystem whose sediments are still contaminated. They represent a potential risk for the biota including benthic and demersal species living in direct contact with the sediment. In this context, the present study aims to survey trends of the health status of the Bilbao estuary based on sediment chemistry and sole (*Solea* spp.) histopathology. Monitoring campaigns were carried out every autumn from 2011 to 2017 along the estuary. Contaminant levels were measured in sediments; liver, gills and gonads of juvenile fish were collected for histopathology. Overall, contaminant levels fluctuated throughout the years, with highest values recorded in the earlier years of the study period. Sole histopathology showed alterations of mild severity. Results permitted to assess the environmental health status of the Bilbao estuary during 7 years, although no clear temporal trend was detected. Longer-term monitoring programmes are necessary to confirm the ecosystem recovery.

Résumé

L’estuaire de Bilbao (SE du golfe de Gascogne) est un écosystème en cours de rétablissement dont les sédiments sont encore considérés contaminés. En effet, ils représentent un risque potentiel pour le biote marin telles que les espèces benthiques et démersales qui vivent en contact direct avec le substrat. L’étude présente a pour objectif de surveiller l’évolution de l’état de santé de l’estuaire de Bilbao en se basant sur l´analyse chimique du sédiment et l’histopathologie de la sole (*Solea* spp.). Des campagnes de surveillance ont été conduites le long de l’estuaire, chaque automne de 2011 à 2017. Les concentrations en contaminants métalliques et organiques ont été mesurés dans le sédiment et des échantillons de foie, branchies et gonades ont été prélevés pour les analyses d’histopathologie. Dans l'ensemble, les niveaux de contaminants variaient au cours des années. Les niveaux les plus élevés ayant été enregistrées durant les premières années de campagne. L’analyse histopathologique chez la sole a montré des lésions de sévérité moyenne. Les résultats ont permis de suivre l’évolution de l’état de santé environnementale de l’estuaire de Bilbao pendant 7 ans bien qu’aucune tendance temporelle n’ait été détectée. Des campagnes de surveillance à long terme sont nécessaire afin de confirmer le rétablissement de l’écosystème.

Laburpena

Bilboko estuarioa (Bizkaiko badian) berreskuratzen dagoen ekosistema da, bere baitan oraindik kutsaturiko sedimentuak dituena. Hauek, biotarekiko arriskutsuak suerta daitezke, espezie bentikoekiko edota sedimentuari erlazio zuzena dituzten espezie demertsalekiko batez ere. Gauzak horrela, lan honen helburua Bilboko estuarioaren osasun egoera ikertzea da.Horretarako, sedimentuen kimika eta mihi-arrainaren (*Solea* spp.) histopatologia aztertuko da. Udazkenetan 2011-2017 bitartean, kutsaduraren jarraipen kanpainak bideratu ziren Bilboko estuarioan zehar. Kutsatzaileen mailak estuarioko sedimentuetan neurtu ziren eta mihi-arrain gazteen gibeletan, zakatzetan eta gonadetan analisi histopatologikoak egin ziren. Oro har, urteetan zehar kutsatzaileen mailak gorabeherak izan zituzten, balio altuenak lehenengo laginketa urteetan zehar eman zirelarik. Mihi-arrainen histopatologiak larritasun baxuko alterazioak erakutsi zituen. Emaitz hauek Bilboko estuarioaren ingurumen osasun maila 7 urteetan zehar aztertzea baimendu du, denborarekiko joerarik garbiak aurkitu ez izan arren. Izan ere, epe luzeko monitorizazio programak beharrezkoak dira ekosistemaren berreskurapen prozesua konfirmatu nahi bada.

1. Introduction

The marine environment is exposed to a large variety of persistent chemicals, which even at low levels can cause adverse effects to the ecosystem (Bernet et al., 1999; Haynes and Johnson, 2000; Moore et al., 2004). Pollution monitoring programmes are therefore developed to survey status and trends of the affected aquatic environments over time. In the Bay of Biscay and the Atlantic Iberian coast, chemical long-term data were recorded for the past years in offshore, coastal and estuarine areas (Besada et al., 2011; 2014; Borja et al., 2011; 2016; Legorburu et al., 2013; 2014). Amongst these areas, the Bilbao estuary (SE Bay of Biscay) attracted great interest for its industrial past and its recent recovery processes (Borja et al., 2006; 2010; 2016; Cajaraville et al., 2016; Irabien et al., 2018). This estuary suffered from intense industrial and domestic pollution of the Bilbao metropolitan area since the 19th century and was consequently highly contaminated (Soto et al., 1995; González-Oreja and Saiz-Salinas, 1998; Orbea and Cajaraville, 2006; Fernández-Ortiz de Vallejuelo et al., 2010; Gredilla et al., 2013). Over the last decades, the industrial decline and improvement of wastewater-treatment in the area enhanced the recovery of both water and sediment quality, as well as the diversity and abundance of the planktonic and benthic communities and fish populations (Saiz-Salinas and González-Oreja, 2000; Cearreta et al., 2004; Borja et al., 2006; García-Barcina et al., 2006; Díez et al., 2009; Fernández-Ortiz de Vallejuelo et al., 2010; Villate et al., 2013; Pouso et al., 2018).

Nevertheless, the recovery of sediment quality is a slow process and thus, some areas of the estuary were still considered moderately toxic (Borja et al., 2015; Cajaraville et al., 2016). Based on sediment analysis, the estuary was chronically impacted by metals, PCBs and PAHs contamination (Montero et al., 2013; Borja et al., 2015). These sediments represented a potential source of pollutants for the surrounding biota (Eggleton and Thomas, 2004).

A particular interest has been drawn towards the use of flatfishes as sentinel species for biomonitoring programmes (Köhler et al., 1992; Myers et al., 1994; Stehr et al., 2003; Dabrowska et al., 2012; Fricke et al., 2012). This is mainly related to their benthic behaviour, which links their general health status to sediment quality (Feist et al., 2004; Lang et al., 2006; Jimenez-Tenorio et al., 2008). In this respect, the common sole (*Solea solea*) and the Senegalese sole (*Solea senegalensis*) are two frequent flatfishes found along the coast of Southern European countries (Quéro et al., 1986). Their suitability as sentinel species for biomonitoring programmes has been proposed in many works. They include laboratory studies (Claireaux et al., 2004; Salamanca et al., 2008; Costa et al., 2010; Ribecco et al., 2012; González-Mira et al., 2016) and short field research carried out in the Mediterranean Sea (Dierking et al., 2009; Sànchez-Nogué, 2013; Siscar et al., 2013; Oliva et al., 2014; Solé et al., 2016), in Portugal (Vinagre et al., 2006; Costa et al., 2013; Gonçalves et al., 2013) and in the Bay of Biscay (Budzinski et al., 2004; Gilliers et al., 2006; Cuevas et al., 2015a, 2015b).

In sole, as well as in other marine organisms, biological responses to contaminants can be measured at different biological organization levels. Tissue-level alterations represent a powerful indicator of medium-term effects of exposure to xenobiotics (Bernet et al., 1999; Feist et al., 2004). In several species of flatfish, including sole, the diagnosis of diseases and histopathological lesions has been successfully linked to exposure to contaminants (Stentiford et al., 2003; Alvarado et al., 2005; Salamanca et al., 2008; Costa et al., 2009). These tissue-level biological responses can be recorded in different organs such as liver, gills and gonads (Hinton and Lauren, 1990; Myers et al., 1994; ICES, 1997; Arellano et al., 1999; Alvarado et al., 2005; 2006; Stentiford and Feist, 2005; Reddy and Waskale, 2013). Their use as target organs for the assessment of biological effects caused by pollution is related to their biological role. Liver, specifically, is involved in xenobiotic transformation, storage and elimination (Hinton et al., 2001; Alvarado et al., 2005). In gills, the contact with water allows a direct uptake of contaminants (Alvarado et al., 2006; 2007; Costa et al., 2009; Lujić et al., 2013). The reproductive cycle and the liability for upcoming generations can be observed through gonad assessment (Blazer, 2002; Solé et al., 2016) since exposure to toxicants can be responsible for gonad histopathological alterations such as intersex (Bateman et al., 2004; Stentiford and Feist, 2005; Minier et al., 2000; Feist et al., 2015; Bizarro et al., 2014) and occurrence of atresia (Blazer, 2002; Reynolds et al., 2003; Ortiz-Zarragoitia et al., 2010).

Considering the above, the objective of the present work was to survey the evolution of the health status of the Bilbao estuary based on data on sediment contamination and histopathological analysis of juvenile *Solea* spp. used as sentinel species during a 7-year survey (2011-2017).

2. Material and Methods

2.1 Study area

The Bilbao estuary is located in the SE Bay of Biscay, on the E Cantabrian Coast (Spain). It is a mesotidal system with a semidiurnal tidal regime and drains a watershed of 1700 km^2, with an annual freshwater inflow of about 36 $m^3 s^{-1}$. The estuary is 22 km long and two areas can be distinguished: an inner part around 15 km long, with a narrow and relatively shallow channel (maximum depth of about 10 m) that crosses the metropolitan area of the city of Bilbao, and an outer part, a semi-enclosed coastal embayment, with a maximum depth of about 30 m (Figure 1). The inner part is highly stratified all year round, but stratification weakens gradually towards the outer part, where, at bottom, salinity values approach those of the surrounding shelf waters.

The original morphology of the estuary has been modified by extracting large intertidal areas and by extensive dredging activities to maintain the navigation on the channel. Due to these changes and according to the European Water Framework Directive (2000/60/EC), the Bilbao estuary is a heavily modified water body.

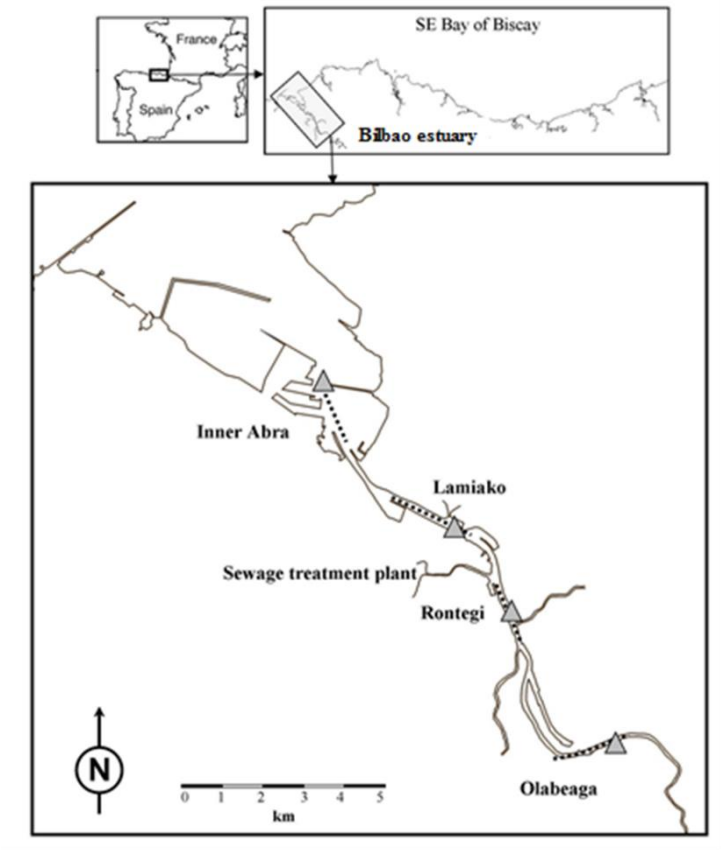

Figure 1. Map of the Bilbao estuary showing the trawling stretches (dotted lines), the location of the sewage treatment plant and the sediment sampling sites (triangles).

From the second half of the 19th century, the industry, urban and port developments in the estuarine municipalities transformed the area into one of the most important economic zones in Spain, mainly due to the development of iron and steel industries. However, the economic development also turned the estuary into a highly polluted coastal area, changing and degrading its morphology and the ecological conditions (Cearreta et al., 2000, 2004). Non-treated industrial wastes and domestic sewage that were discharged directly into the estuary (Belzunce et al., 2004; Borja et al., 2006) caused intense pollution in its waters and sediments (Borja et al., 2010) and a great degradation of the biological communities (Saiz-Salinas and González-Oreja, 2000).

In 1979, a sewerage scheme for the area was approved; it consisted of more than 300 km of interceptors and a central wastewater treatment plant (Galindo WWTP). The main objective of this scheme was the recovery of the aquatic life in all the system, as well as the rehabilitation, for the public use, of the beaches located in the outer part of the estuary. From 1990, waters were being cleaned with a physicochemical (primary) treatment. The population served by the sewerage system and the volume of water treated progressively increased. In addition, a biological (secondary) treatment started in 2001.

The implementation of the sewerage scheme and the decline of industrial activities at the end of the 20th century allowed the progressive recovery of the estuarine waters (Cajaraville et al., 2016), with a consequent improvement in the ecological quality (Borja et al., 2016), biological value (Pascual et al., 2012) and positive effects in cultural ecosystem services such as recreational fishing and beach recreation (Pouso et al., 2018a, 2018b).

2.2 Sampling campaigns

Sampling campaigns were carried out along the Bilbao estuary every autumn (Sept.-Oct.) from 2011 to 2017. At the beginning of the study, from 2011 to 2013, 30 fish specimens were collected in the innermost part of the estuary and 30 others in the outermost part (Figure 1). As primary results showed no significant difference between general health status of individuals from different stretches based on histopathological analysis (data not shown), 30 individuals were collected throughout the whole estuary since 2014 (ESM: 1). *Solea* spp., including common sole (*Solea solea*) and Senegalese sole (*Solea senegalensis*), were collected by bottom trawling (beam trawl, 2.5 m wide, 40 mm mesh size and 14 mm mesh size cod end; towed for 10 min at 1.5 knots) at 4 stretches from the head to the mouth of the estuary and were grouped together in order to consider the whole estuary as one same sampling area. In total, 268 fish were caught throughout the 7 years of campaigns. Immediately after sampling, fish were anaesthetised by use of benzocaine dissolved in water and transported to the laboratory for dissection.

Each year, sediment samples were collected by a Van Veen grab at four sampling sites situated along the estuary (obtaining in total 28 sediment samples) for contaminant determination (Figure 1). Since fish samples were brought together as originating from one same sampling site, average of chemical data obtained from the four sediment samples was calculated each year for each of the contaminants studied.

2.3 Sediment contamination levels and toxicological significance

Metal (Cd, Cr, Cu, Hg, Ni, Pb and Zn) content was measured in triplicate in acid extracts from the fine fraction of the sediments (<63 mm). In brief: dried sediment was digested in an acid mixture (2HCl:1HNO_3) using microwave system (MARS 5 Xpress CEM Corporation Instrument). Afterwards, metal levels were determined by Atomic Absorption Spectrometry, AAS (AAS800 Perkin Elmer): Cd was analysed by THGA graphite furnace, using Zeeman background correction; Cr, Cu, Ni, Pb and Zn were determined in an air acetylene flame; finally, total Hg was measured by quartz furnace AAS following cold vapour method. Analytical accuracy was checked by the PACS-2 reference material (National Research Council of Canada, NRC) and the measured values were found to be within the certified range. Organic compounds such as polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) were also determined. Sediment samples (5-10 g) were pre-concentrated with a mixture of solvents (pentane: dicloromethane; 50:50) by Accelerated Solvent Extraction, ASE (200 system DIONEX). Organic extract was purified by Gel Permeation Chromatography (GPC) and different

extracts were collected, evaporated and reconstituted by isooctane for PCBs or by ethyl acetate for organochlorides. 8 ml of sulphuric acid were added to PAHs extract and then it was centrifuged. Organic phases were collected and determined by Gas Chromatography-Mass Spectrometry, GC-MS (Agilent 6890 GC coupled with an Agilent 5973 MSD instrument).

Contaminant levels were compared with the values of the Effect Range Low (ERL) and Effect Range Median (ERM) for metals, PCBs and PAHs (Long et al., 1995). The contaminant potential to cause adverse biological effects was assessed through the estimation of Sediment Quality Guideline Quotients (SQG-Qs). The SQG-Qs were calculated as the ratio between the content of individual chemicals and their respective ERM value. The sediments were then ranked as proposed by MacDonald et al. (2004) according to their toxicological risk for each class of contaminants (metals, PCBs and PAHs): SQG-Q < 0.1 as non-impacted sediments; 0.1 - 1 as moderately impacted and > 1 as strongly impacted. In this approach, the mean of individual SQG-Qs of each chemical group was calculated.

2.4 Biometric parameters

Biometric data (total wet weight (g) and total length (cm)) were used to calculate the condition factor of each individual (K) = total weight × 100 / total length^3 (g.cm^{-3}) (Ricker, 1975).

2.5 Dissection and histological procedure

Liver and gonad samples were collected every autumn from 2011 to 2017, while gill samples were obtained from 2013 to 2017. All biological samples were excised and immediately fixed in 4% neutral buffered formaldehyde (Martoja and Martoja, 1967). Formalin-fixed samples were dehydrated in a graded series of ethanol, cleared and embedded in paraffin (Leica ASP 300S). Sections of 5 µm were obtained using a rotary microtome (Leica RM2125 RTS) and stained with hematoxylin-eosin (H&E; Martoja and Martoja-Pierson, 1970). Histological examination of liver, gill and gonad samples was carried out under a light microscope (Nikon Eclipse E200).

2.6 Gamete developmental stages

Gonad samples were analysed at the microscope for gender and gamete developmental stages determination. Gamete developmental stages in males were determined according to García-López et al. (2006) and were classified in five stages as follows: Stage I (early spermatogenesis); Stage II (mid spermatogenesis); Stage III (late spermatogenesis); Stage IV (mature); Stage V (recovery). The identification of gamete developmental stages for females was mainly based on Murua and Motos (2006). Stages were classified as follows: Stage I (growth); Stage II (early vitellogenesis); Stage III (late vitellogenesis); Stage IV (maturation).

2.7 Histopathological analysis

The prevalence of histopathological alterations (% = [number of cases/total cases analysed] ×100) was estimated per sampling year for liver and gills and per sampling year and gender for gonads.

Alterations were classified into four categories for liver, gills and gonad: (1) circulatory disturbances; (2) inflammatory responses; (3) regressive changes; (4) progressive changes.

For each lesion categorie, semi-quantitative histopathological indices were measured according to Bernet et al. (1999) and adapted by Costa et al. (2009). A global histopathological index was calculated for each individual and each organ:

$$I_h = \sum_1^j w_j a_{jh}$$

where w_j is the weight of the j^{th} histopathological alteration and a_{jh} the score given to the j^{th} alteration for the individual *h*. Weights were given to each lesion based on their pathological importance as: (1) minimal; (2) moderate and (3) severe. Scores were classified from 0 to 6 according to the level of dissemination of the alteration in the organ where 0 is absence and 6 is high degree of dissemination.

2.8 Statistical analysis

Statistical analyses were carried out using IBM SPSS Statistics Base 22.0. Homogeneity of variance (Levene´s test) and normality of data (Kolmogorov-Smirnov's test) were tested before statistical analysis. For non-normal data set, the non-parametric Kruskal-Wallis test and Mann-Whithney U test were used to analyse differences in biometric data and in histopathological data throughout the years. The Chi-squared test was used to compare histopathological lesion prevalence between years and between genders. The non-parametric Spearman's rank-order (R) was used to assess correlations between the different surveyed variables and between variables and year. The level of significance considered for all analyses was $\alpha = 0.05$.

3. Results

3.1. Sediment contamination levels and toxicological significance

Contaminant levels were recorded yearly in sediment samples collected in the Bilbao estuary (Table 1). There was no significant trend between contaminant content (annual averages for the whole estuary) and years of the study ($p>0.05$, $n=7$). Nevertheless, the highest levels of PAHs (26464.69 μg kg^{-1}) and PCBs (582.55 μg kg^{-1}) were observed in 2011, while the lowest levels were found in 2017 (PAHs: 5027.50 μg kg^{-1} and PCBs: 74.53 μg kg^{-1}). For metals, a higher temporal variability was recorded when compared with organic contaminants. Several metals such as Cu, Fe, Hg, Pb and Zn showed two peaks in concentrations, one in 2011 and the other one in 2016. As for the toxicological significance (SQG-Qs), sediments from the Bilbao estuary were moderately or strongly

Table 1. Annual mean of metals (mg/kg in d.w.) and organic contaminants content (µg/kg in d.w.) and toxicological significance (SQG-Qs) of sediments derived from the four stations located along the Bilbao estuary during 2011 to 2017. N: non-impacted; M: moderately impacted; S: strongly impacted.

Years	Cd	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Zn	SQG_Q metals	∑PCBs	SQG_Q PCBs	∑PAHs	SQG_Q PAHs
2011	1.25	89.4	266.9	45230	5.90	436.4	30.90	515.0	626.9	2.02 (S)	582.6	3.24 (S)	26465	0.59 (M)
2012	0.71	69.1	66.2	34784	1.10	252.3	38.55	81.6	234.1	0.54 (M)	179.3	1.00 (M)	2699	0.06 (N)
2013	1.81	93.7	122.3	39364	1.41	405.6	61.88	187.7	307.1	0.81 (M)	127.7	0.71 (M)	16612	0.37 (M)
2014	0.84	82.6	80.0	35369	0.79	365.7	38.10	99.8	266.2	0.51 (M)	246.6	1.37 (S)	3867	0.09 (N)
2015	0.81	81.3	89.3	36765	0.49	334.8	40.58	128.1	335.0	0.50 (M)	115.9	0.64 (M)	12060	0.27 (M)
2016	3.50	134.3	266.8	47003	3.48	387.5	46.75	703.0	938.5	1.86 (S)	308.7	1.71 (S)	11290	0.25 (M)
2017	1.24	72.3	77.0	34596	0.58	367.3	34.50	88.3	285.5	0.46 (M)	74.5	0.41 (M)	5028	0.11 (M)

Note: ∑PCB: is the sum of 28, 52, 101, 118, 138, 153 and 180 congeners; ∑PAH: is the sum of fluorene, naphthalene, anthracene, dibenz(a,h)anthracene, acenaphthene, acenaphthylene, phenantrene, pyrene, chrysene, fluoranthene, benzo(a)anthracene, benzo(a)pyrene.

impacted by metals and PCBs, and non-impacted or moderately impacted by PAHs. The toxicological risk fluctuated throughout the study period; nevertheless, metals and PCBs were the contaminants most likely to cause adverse effects as, according to MacDonald et al. (2004), they were ranked as strongly affected (SQG-Qs >1) in several years.

3.2. Biometric parameters

A summary of annual *Solea* spp. captures and corresponding biometric data is shown in Table 2. Overall, soles measured 23.7 ± 4.5 cm and weighted 130.8 ± 96.8 g. Individuals were significantly smaller in 2011 (20.3 ± 3.2 cm; 77.9 ± 36.5 g) and larger in 2016 (29.2 ± 4.5 cm; 249.0 ± 140.5 g). *K* was significantly lower in soles from 2015 and 2017 than in soles from the rest of campaigns.

Table 2. Mean and standard deviation of biometric parameters in *Solea* spp. collected in the Bilbao estuary every autumn from 2011 to 2017. Different letters indicate significant differences between years ($p<0.05$).

Sampling year	n	Sex ratio (M:F)	N.D	Length (cm)	Weight (g)	*K*
2011	64	1:2.0	17	20.3 ± 3.2^{a}	77.9 ± 36.5^{a}	0.9 ± 0.1^{a}
2012	53	1:1.4	3	23.8 ± 5.1^{b}	134.6 ± 123.3^{bc}	0.9 ± 0.1^{a}
2013	31	1:1.4	2	25.9 ± 3.9^{c}	175.4 ± 86.9^{de}	0.9 ± 0.1^{b}
2014	30	1:0.9	0	22.3 ± 1.5^{ad}	96.3 ± 22.1^{bf}	0.9 ± 0.1^{a}
2015	30	1:1.0	2	23.5 ± 2.5^{bdf}	108.2 ± 31.9^{cf}	0.8 ± 0.2^{c}
2016	30	1:2.1	5	29.2 ± 4.5^{e}	249.0 ± 140.5^{d}	0.9 ± 0.3^{a}
2017	30	1:1.1	0	24.8 ± 2.7^{cf}	129.8 ± 49.9^{ce}	0.8 ± 0.1^{c}
Total	268	1:1.4	29	23.7 ± 4.5	130.8 ± 96.8	0.9 ± 0.2

n: sample size; N.D: number of individuals where gender could not be determined; K: condition

3.3. Gamete developmental stages

Sex ratio and gamete developmental stages were recorded for each gender in soles collected each sampling year (Table 3). Throughout the 7 years of campaigns, the histological analysis of gonads mainly showed fish in early stages of gamete development. Similar gamete developmental stages were observed among years in males and females. Males mostly showed early (74.7%) and mid spermatogenesis (22.1%) stages. Cases of mature (2.1%) and recovery (1.1%) stages were sporadically identified. Over the 7 years of campaigns, 97.8% of females showed gametes in growth stage, but residual cases of late vitellogenesis (0.7%) and maturation (1.5%) were also identified.

Table 3. Gamete developmental stages (%) determined in Solea spp. collected in the Bilbao estuary every autumn from 2011 to 2017.

	2011	2012	2013	2014	2015	2016	2017	Total
Males	n=16	n=21	n=12	n=16	n=8	n=8	n=14	n=95
Stage I	75.0	95.2	66.7	50.0	75.0	62.5	86.7	74.7
Stage II	25.0	4.8	25.0	50.0	25.0	25.0	6.7	22.1
Stage III	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Stage IV	0.0	0.0	0.0	0.0	0.0	12.5	6.7	2.1
Stage V	0.0	0.0	8.3	0.0	0.0	0.0	0.0	1.1
Females	n=32	n=29	n=17	n=13	n=11	n=17	n=16	n=135
Stage I	100.0	96.6	94.1	100.0	100.0	94.1	100.0	97.8
Stage II	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Stage III	0.0	3.4	0.0	0.0	0.0	0.0	0.0	0.7
Stage IV	0.0	0.0	5.9	0.0	0.0	5.9	0.0	1.5
Indeterminate	n=16	n=3	n=2	n=1	n=11	n=5	n=0	n=38

n: sample size

3.4. Hepatic, gill and gonad histopathological analysis

The liver of *Solea* spp. was characterised by a bi-layer of hepatocytes arranged around sinusoids showing the typical cord-like structure of fish liver (Figure 2A). The general cell appearance varied from eosinophilic to clear cytoplasm. It was also common to observe the exocrine pancreas distributed in the liver tissue and various bile ducts typically delimited by connective tissue.

The most frequent liver histopathological alterations identified in *Solea* spp. were inflammatory and progressive changes followed by regressive and circulatory disturbances (Table 4). The most prevalent circulatory disturbance was hyperaemia (Figure 2B) characterised by blood congestion and sinusoidal swelling. This hepatic lesion was significantly more abundant in 2011 and 2012 (46.2-54%) than in the rest of the years (3.7-29%). Only few cases of haemorrhage ranging from 0% in the last three years to 9.6% in 2012 were detected and were restricted to small foci. Melanomacrophage centres (MMCs) were the most common inflammatory responses (Figure 2C). In 2011, 2015 and 2016 the prevalence of MMC exceeded 84%. Regressive changes were neither frequent nor severe. Few cases of necrosis ranging from 6.3% in 2011 to 38.7% in 2013 (Figure 2D) and nuclear pleomorphism presenting a mean value of 15.4% were identified. Among progressive changes, fat vacuolation of hepatocytes, the most prevalent lesion identified, was homogeneously distributed throughout the parenchyma (Figure 2E). The lowest prevalence of fat vacuolation of hepatocytes was recorded in 2011 (14.3%) while the highest levels were observed in 2017 (90%). The highest prevalence of *spongiosis hepatis* was detected in 2012 (23.1%) and was absent from 2014 onwards. The frequency of concentric periductal fibrosis (CPF) of bile ducts decreased from a maximum value in 2011 (30.2%) to a minimum value in 2016 (3.3%). From the seven-year survey, one case of hepatic malignant neoplasm was detected in 2016 in a female sole (Figure 2G-I).

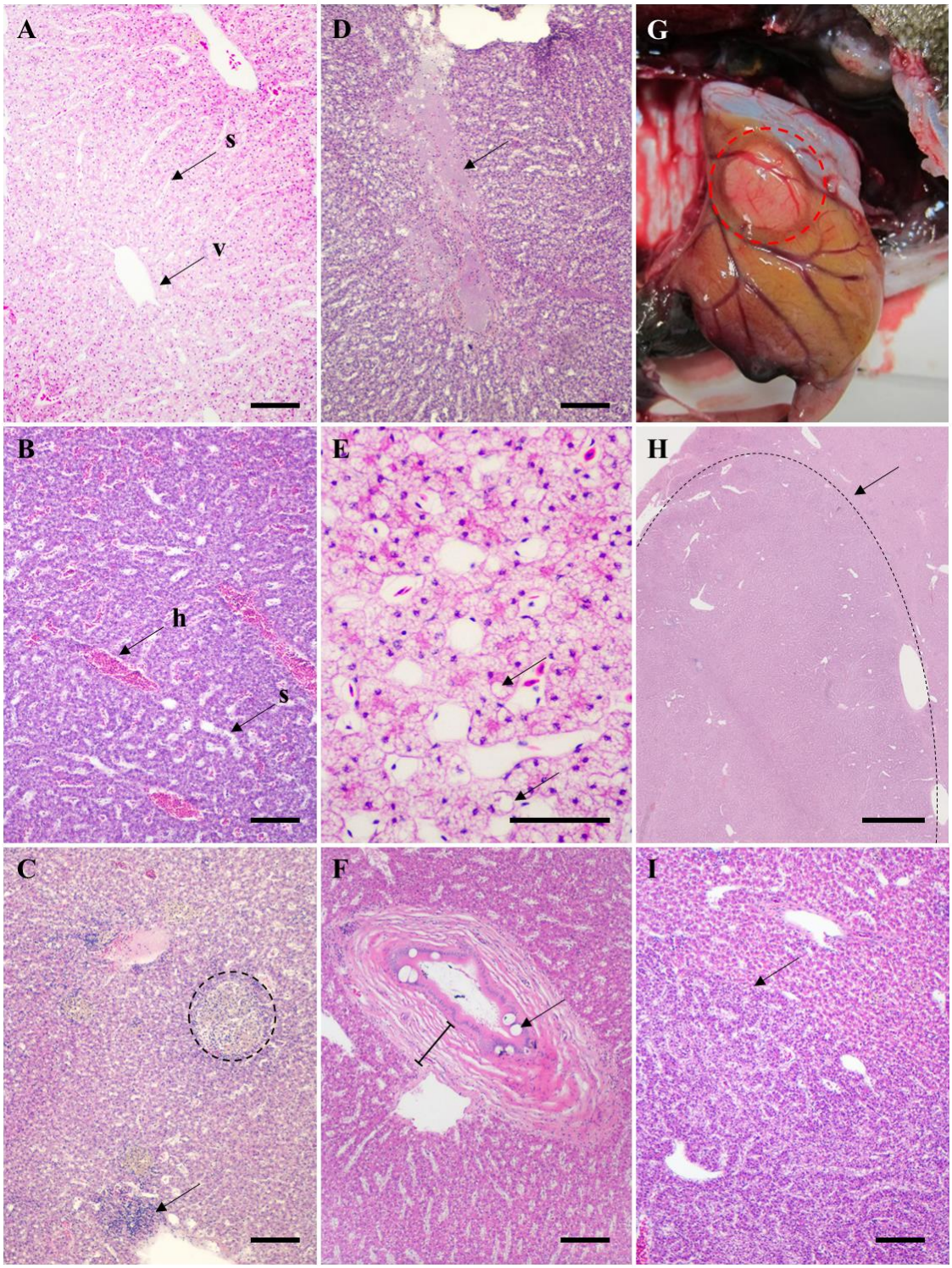

Figure 2. Hepatic sections of *Solea* spp. stained with hematoxylin-eosin. (A) Hepatic tissue showing the bi-layer arrangement of hepatocytes around sinusoids; s: sinusoid; v: vein (B) Hepatic hyperaemia with high abundance of erythrocytes in blood vessels; h: hyperaemia; s: sinusoid. (C) Hepatic tissue showing melanomacrophage centres (dotted circle) and lymphocytic infiltration (arrow). (D) Severe case of hepatic necrosis (arrow). (E) Hepatic fat vacuolation showing multiple vacuoles (arrow). (F) Hepatic tissue presenting severe concentric periductal fibrosis (segment) and hydropic vacuolation of bile duct epithelial cells (arrow). (G-I) Hepatic nodule identified in a female *S. solea* collected in the Bilbao estuary in autumn 2016 at (G) macroscopic level and (H, I) histological level showing the delimitation of the nodule (arrow). Scale bar (a-d, f, i): 100 µm; scale bar (e): 50 µm; scale bar (h): 1 mm.

Table 4. Prevalence (%) of hepatic alterations in *Solea* spp. collected every autumn from 2011 to 2017. Different letters indicate significant differences between years ($p<0.05$).

Hepatic alterations	*w*	2011 n=63	2012 n=52	2013 n=31	2014 n=28	2015 n=30	2016 n=27	2017 n=30
Circulatory disturbances								
Haemorrhage	1	6.3	9.6	3.2	3.6	0.0	0.0	0.0
Hyperaemia	1	54.0 ^{a}	46.2 ^{ab}	29.0 ^{bc}	21.4 ^{cd}	10.7 ^{cd}	3.7 ^{d}	10.0 ^{cd}
Inflammatory responses								
MMCs	1	84.1 ^{a}	57.7 ^{b}	58.1 ^{bc}	78.6 ^{ac}	89.3 ^{a}	92.6 ^{a}	73.3 ^{abc}
Lymphocytic infiltration	2	42.9	46.2	38.7	46.4	35.7	48.2	50.0
Regressive changes								
Necrosis	3	6.3 ^{a}	13.5 ^{ab}	38.7 ^{c}	14.3 ^{ab}	25.0 ^{bcd}	22.2 ^{bc}	10.0 ^{ad}
HNP	2	20.6	15.4	16.1	10.7	3.6	14.8	26.7
Progressive changes								
Spongiosis hepatis	2	6.3 ^{a}	23.1 ^{b}	3.2 ^{a}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}
FV of hepatocytes	1	14.3^{a}	44.2 ^{b}	77.4 ^{ce}	50.0 ^{bd}	60.7 ^{bcd}	70.4 ^{cde}	90.0 ^{e}
HV of epithelial cells of bile duct	2	7.9	5.8	6.5	7.1	10.7	7.4	3.3
CPF of bile ducts	2	30.2 ^{a}	21.2 ^{a}	19.4 ^{ab}	14.3 ^{ab}	3.6 ^{b}	11.1 ^{ab}	3.3 ^{b}
Neoplastic lesions								
Malignant tumour		0.0	0.0	0.0	0.0	0.0	3.7	0.0

w: biological significance of each alteration; n: sample size for hepatic histopathology; MMCs: Melanomacrophage Centres; HNP: Hepatocellular Nuclear Pleomorphism; FV: Fat Vacuolation; HV: Hydropic Vacuolation; CPF: Concentric Periductal Fibrosis.

Hepatic histopathological indices calculated in *Solea* spp. for each sampling year are presented in Figure 3A. The highest total hepatic histopathological indices were measured in the earlier years of the study period with a maximum value detected in 2013 (10.00 ±0.86). In 2014, a significant decrease was observed in the index (7.71 ±0.97) and values were maintained lower than in 2013 throughout the following years with no significant differences. The highest total hepatic histopathological index recorded in 2013 was mainly related to regressive and progressive changes, which reached their highest levels in the year (2.97 ±0.23 and 3.68 ±0.68, respectively). Amongst the other categories of lesion, the highest levels of circulatory disturbances and inflammatory responses were recorded in 2011 (1.46 ±0.23 and 4.16 ±2.95, respectively). Circulatory disturbances showed a decreasing temporal trend, with values in 2011 significantly different from the following years.

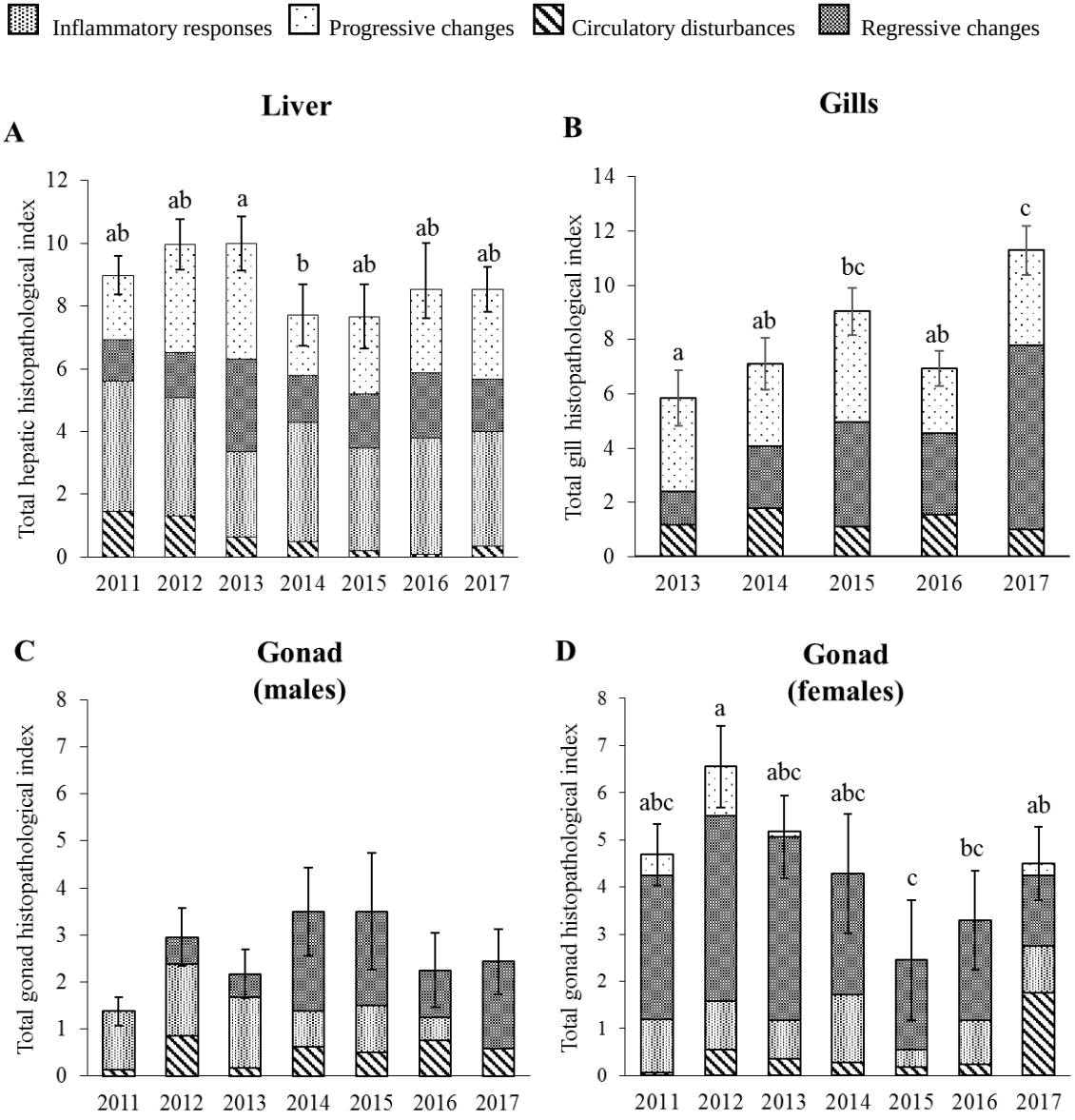

Figure 3. Mean and standard error of total histopathological index and corresponding lesion categories calculated in Solea spp. for each sampling year in (A) liver, (B) gill and (C) male and (D) female gonad. Different letters indicate significant differences between years ($p<0.05$).

Amongst the histopathological alterations identified in gills, regressive changes were the most frequent (Table 5). Epithelial lifting was significantly more abundant in 2017 (89.3%) than in any other year and epithelial desquamation was significantly more frequent in *Solea* spp. from 2015 to 2017 (66.7%-89.7%) than in earlier years (23.1-40.7%). Amongst progressive changes, epithelial hyperplasia was the most prevalent lesion (36.7-72.4%). Hypertrophy of pavement cells was significantly higher in 2013 (19.2%) than in 2014, 2015 and 2017 (0%, 3.4%, and 0%, respectively). Cases of lamellar fusion and hypertrophy of chloride cells were rarely identified (<10%). The only circulatory disturbance observed in gills was aneurysm, showing a stable prevalence (46.4-76.7%). No inflammatory responses were recorded in gills.

Table 5. Prevalence (%) of gill histological alterations identified in *Solea* spp. collected every autumn from 2013 to 2017. Different letters indicate significant differences between years ($p<0.05$).

Gill alterations	*w*	2013 n=26	2014 n=27	2015 n=29	2016 n=30	2017 n=28
Circulatory disturbances						
Aneurysm	1	53.8	70.4	55.2	76.7	46.4
Regressive changes						
Epithelial lifting	1	23.1^{a}	29.6^{a}	24.1^{a}	43.3^{a}	89.3^{b}
Epithelial desquamation	1	23.1^{a}	40.7^{a}	89.7^{c}	66.7^{c}	82.1^{c}
Progressive changes						
Lamellar fusion	1	0.0	7.4	6.9	10.0	10.7
Epithelial hyperplasia	2	53.8	51.9	72.4	36.7	53.6
Hypertrophy of pavement cells	1	19.2^{a}	0.0^{b}	3.4^{b}	10.0^{ab}	0.0^{b}
Hypertrophy of chloride cells	2	7.7	0.0	0.0	6.7	7.1

w: biological significance of each alteration; n: sample size for gill histopathology

Total gill histopathological indices were calculated in *Solea* spp. for each sampling year (Figure 3B). The highest total gill histopathological index recorded in fish from 2017 (11.29 ±0.90) was significantly higher from indices detected in fish collected in 2013 (5.85 ±1.02), 2014 (7.11 ±0.95) and in 2016 (6.93 ±0.66). The lesion category that contributed the most to achieve the highest total gill histopathological index was regressive changes (Figure 3B).

The frequency of histopathological lesions identified in gonads was measured for each gender and each sampling year (Table 6). Hyperaemia was the only circulatory disturbance detected and was limited to small areas for both genders. Hyperaemia frequencies varied from 3.1% in females collected in 2011 to 62.5% in females sampled in 2017. MMCs were the most frequent inflammatory alteration followed by lymphocytic infiltration and the presence of granulomatous tissue. The frequency of MMCs decreased in both genders from 2011 onwards. No progressive changes were detected in males, but a few cases of lipid droplets accumulated in pre-vitellogenic oocytes were shown in female gonads. Regressive changes were identified as necrotic foci (0-38.5%) and pyknotic oocytes/spermatocytes (0-50%). Additionally, female gonads showed few cases of oocyte atresia (0-41.4%) in early gametogenic stages. No cases of intersex were detected in male gonads from the present study.

Total gonad histopathological indices calculated in *Solea* spp. per gender and year are presented in Figure 3C-D. Total male gonad histopathological indices did not significantly differ throughout the sampling years. In females, the highest total gonad histopathological index was measured in fish from 2012 (6.55 ±0.87) and was related to the increase in prevalence of lipid accumulation, hyperaemia, pyknotic nuclei and atresia.

Table 6. Prevalence (%) of gonad histological alterations identified in males and females *Solea* spp. collected every autumn from 2011 to 2017. NA: Not Applied. Different letters indicate significant differences between years for the same gender ($p<0.05$). Asterisks indicate significant differences between genders of the same sampling year.

		2011		2012		2013		2014		2015		2016		2017	
Gonad alterations	*w*	M n=16	F n=32	M n=21	F n=29	M n=12	F n=17	M n=16	F n=13	M n=8	F n= 11	M n= 8	F n= 17	M n= 14	F n= 16
Circulatory disturbances															
Hyperaemia	1	6.3	3.1^{a}	38.1	27.6^{b}	8.3	17.6^{ab}	31.3	15.4^{ab}	25.0	9.1^{ab}	37.5	11.8^{ab}	28.6	62.5^{c}
Inflammatory responses															
MMCs	1	62.5^{a}	56.3^{a}	47.6^{a}	34.5^{b}	58.3^{a}	$17.6^{bc,*}$	6.3^{b}	0.0^{c}	0.0^{b}	0.0^{c}	25.0^{ab}	$0.0^{c,*}$	0.0^{b}	0.0^{c}
Granulomatous tissue	2	0.0	0.0	0.0	6.9	0.0	11.8	6.3	15.4	0.0	0.0	0.0	5.9	0.0	6.3
Lymphocytic infiltration	2	0.0	0.0^{a}	14.3	$0.0^{*, a}$	0.0	0.0^{a}	6.3	23.1^{b}	25.0	9.1^{ab}	0.0	17.6^{b}	0.0	18.8^{b}
Regressive changes															
Pyknotic spermatocytes/oocytes	2	0.0^{a}	$28.1^{a,*}$	0.0^{a}	$31,0^{a,*}$	0.0^{a}	17.6^{ab}	43.8^{b}	$0.0^{b,*}$	50.0^{b}	$0.0^{b,*}$	25.0^{ab}	$0.0^{b,*}$	35.7^{b}	0.0^{b}
Necrosis	3	0.0	0.0^{a}	9.5	3.4^{ad}	8.3	11.8^{ac}	6.3	38.5^{b}	0.0	27.3^{bcd}	0.0	23.5^{bcd}	7.1	25.0^{bc}
Atresia	3	NA	34.4^{ac}	NA	$41.4^{a,*}$	NA	$35.3^{ac,*}$	NA	7.7^{c}	NA	0.0^{bc}	NA	5.9^{bc}	NA	0.0^{bc}
Progressive changes															
Lipids in oocytes	1	NA	21.9^{ab}	NA	37.9^{a}	NA	5.9^{b}	NA	0.0^{b}	NA	0.0^{b}	NA	0.0^{c}	NA	0.0^{c}

M: Males; F: Females; n: sample size; w: biological significance of each alteration; MMCs: Melanomacrophage Centres.

The maximum values found in 2012 were significantly higher than indices recorded in females collected from 2015 (2.45 ±1.27) and 2016 (3.29 ±1.04). A secondary peak was observed in 2017 (4.50 ±0.79) and was significantly higher than the histopathological index measured in 2015. The high index value recorded in females from 2017 was mainly related to circulatory disturbances. The value of this lesion category was significantly higher than any other year.

3.5. Correlation analysis between sediment contaminant levels and histopathological alterations

There was not any direct correlation between the annual mean of the total liver, gills and gonad histopathological indices and sediment contaminant levels ($p>0.05$, $n=7$ for liver and gonad; $n=5$ for gills). However, total liver histopathological index and female total gonad histopathological index were significantly and positively correlated ($R=0.8571$, $p=0.035$, $n=7$).

4. Discussion

The 7-year biomonitoring study carried out in the Bilbao estuary showed fluctuations in sediment contaminant levels and general health status of *Solea* spp. According to SQG-Qs based on Long et al. (1995), sediment contaminant levels were classified as moderately to strongly impacted by metals and PCBs and non-impacted to moderately-impacted by PAHs. The magnitude of liver and gonad histopathological alterations was comparable to previous levels recorded in adult *S. solea* from the Basque coast (offshore), which was considered a low-impacted area (Cuevas et al., 2015a, 2015b). In the case of gills, there was no histopathological data available from field studies using juvenile *Solea* spp. of similar size. The histopathological alterations were not in accordance with fluctuations observed in chemical levels of the same year. The lack of direct relation between chemical and biological data could be related to the time scale of the recovering processes in aquatic ecosystems, where the improvement of the health status, after a long exposure to contaminants, can be slow and incomplete, depending on the severity and duration of pollution events (Mason, 1988; Orbea and Cajaraville, 2006; Garmendia et al., 2011). On the other hand, considering the short length of the Bilbao estuary (22 km) and the movement of soles throughout the area, histopathology in soles may not reflect site-specific sediment pollution levels. Furthermore, similar observations have been made using small sedentary fish (gobies) and sediment contaminant levels in previous research (Cuevas et al., 2016).

The majority of previous field studies using *Solea* spp. lasted from only few months to few years (Budzinski et al., 2004; Laubier et al., 2004; Dierking et al., 2009; Oliva et al., 2012a, 2012b, 2013, 2014; Gonçalves et al., 2013; Cuevas et al., 2015a; Solé et al., 2016); long-term biomonitoring programmes using *Solea* spp. are scarce. To our knowledge, the present work is the longest biomonitoring programme until now (7 years) using juvenile *Solea* spp. as sentinel species. These long-term monitoring programmes combining chemical and biological approaches are essential for the assessment of human pressure

on the ecosystem and for the consequent management of future actions (Borja et al., 2016; Parera et al., 2018).

Chemical data recorded throughout the years of campaign showed fluctuations in contaminant levels from sediment of the Bilbao estuary. Although no clear temporal trend was recorded in sediment contamination, data from the year 2011 described higher contaminant levels than the following years. This is in agreement with previous studies in this estuary describing the temporal improvement of environmental quality measured in terms of metal concentrations (Fernández-Ortiz de Vallejuelo et al., 2010). However, high variability of contaminant levels was recorded in sediment of the Bilbao estuary throughout the 7 years of survey. These fluctuations could be related to natural physical and hydrological processes commonly observed in estuarine areas (Kennish, 1998; Chakraborty et al., 2014) and/or the influence of urban, commercial and industrial activities (Johnston, 1981; Spencer, 2002; Durán and Nieto, 2012). Additionally, we cannot exclude that sporadic peaks in sediment contamination levels may be related to sediment remobilization works occurring in the estuary (Rodriguez-Iruretagoiena et al., 2016). Even though the overall contamination of the estuary reflected an improvement in environmental quality, sediment chemistry still represented a potential risk for the aquatic biota, including for benthic species.

For the assessment of the general health status of the biota from the Bilbao estuary, juvenile soles were collected each year, in the same area and during the same season. These homogeneous sampling conditions are essential in biomonitoring programmes and permit to reduce the potential influence of confounding factors such as individuals' age, food availability and seasonality (ICES, 1997; Bernet et al., 1999). Indeed, based on biometric data, individuals recorded for the present study were juveniles of similar age and were approximately less than 2 years old (Gonçalves et al., 2013; Morat et al., 2014; Cuevas et al., 2015a). *S. solea* spends this period of life in estuaries where they grow until maturation, implying that they have not yet reached deeper coastal areas to spawn. *S. senegalensis* stay in estuarine areas for the first 8 month of their life and migrate to coastal areas to grow; they reach maturation after three years (Le Pape et al., 2003). Accordingly, juvenile *Solea* spp. collected in autumn mainly showed immature gonads since less than 1.5% of individuals were mature. Thus, the general health status of juvenile soles collected from the Bilbao estuary are not influenced by the reproduction process and individuals from different sampling years are in comparable life stage. Therefore, less than 2 year old juvenile *Solea* spp. collected yearly from the Bilbao estuary can be used to assess and survey the general health status of the estuary.

In the present study, the use of liver, gills and gonad as target organs for the assessment of the general health status of juvenile soles permitted to integrate biological responses to both direct and indirect exposure to contaminants. Histopathological lesions identified in the three organs throughout the 7 years of campaign were of mild severity, although one case of hepatic malignant neoplasm was detected. Overall, levels of histopathological indices were comparable to previous studies showing non-severe cases of contamination

(Costa et al., 2011; Cuevas et al., 2015a, 2015b). Changes detected in liver and gonad histopathological indices throughout the years were similar to changes described in previous work from the same area (Cuevas et al., 2016). Histopathological data obtained in the present work suggest a moderate effect of contaminants on juvenile soles with a tendency to recover from the past contamination observed in the Bilbao estuary.

Hepatic histopathology is considered a robust approach to assess biological effects of xenobiotics in fish (Bucke et al., 1996; Stentiford et al., 2003; Feist et al., 2004; Lang et al., 2006; Fricke et al., 2012). In the present study, most of the prevalence of hepatic alterations were similar to those registered in previous works using juvenile *S. senegalensis* from a contaminated estuary in Portugal (Oliva et al., 2013) or with adult *S. solea* from the Basque continental shelf (Cuevas et al., 2015a). The prevalence of MMCs was higher than the levels detected in adult soles collected offshore (Cuevas et al., 2015a) which could indicate a greater activation of the immune system in juvenile soles (Bucke et al., 1992; Fricke et al., 2012). Similarly, frequencies of CPF of bile ducts measured in juvenile *Solea* spp. in 2011 and 2012 in this study were higher than the levels previously detected in adults collected along the Basque continental shelf (Cuevas et al., 2015a). Differences in lesions prevalence between juveniles and adults suggest a higher sensibility of young individuals to environmental conditions.

Previous studies based on hepatic histopathological approaches in flatfish identified cases of neoplastic lesions in adults (Vethaak et al., 1996; Lang et al., 2006; Stentiford et al., 2009, 2010; Feist et al., 2015). However, cases of neoplasms in fish are not expected in juveniles less than 3 years old (Myers et al., 1992). Yet, one case of malignant neoplasm was detected in 2016. The lifetime of juvenile *Solea* spp. could imply early exposure to xenobiotics present in estuaries, which may justify the eventuality of tumour development in relatively young flatfish individuals (Köhler, 2004). Although the lesion has been detected at very low prevalence (1 case out of 268 fish), this is the first case of malignant neoplasm detected in a *Solea* spp. from the Bilbao estuary.

The highest values of the hepatic histopathological index were observed in earlier years (2011-2013); further on, a significant decrease was observed in the index in 2014 and values were maintained lower than in 2013 throughout the following years. This trend suggests an improvement in environmental conditions in the estuary. A similar trend in hepatic histopathological indices was also observed previously in *Pomatoschistus* spp. from the Bilbao estuary (Cuevas et al., 2016). Overall, hepatic histopathological index values were comparable to reported data on *Solea* spp. that had been associated to non-severe cases of contamination (Costa et al., 2011; Cuevas et al., 2015a, 2015b).

The lesions identified in gills from soles collected for the present work were of the same type as those reported in fish from contaminated sites (Stentiford et al., 2003; Camargo and Martinez, 2007; Santos et al., 2014) and in fish exposed to pesticides (Cengiz, 2006), contaminated sediments (Martins et al., 2015) or organic toxicants (Rosety-Rodríguez et al., 2002). Epithelial lifting, hyperplasia and hypertrophy of the gills epithelial cells are commonly interpreted as tissue-level responses to contaminant exposure that enhance the

barrier against the entry of environmental xenobiotics through the gills (Martinez et al., 2004). As counterpart, by increasing the distance between water and blood, these lesions affect respiratory and osmoregulatory functions, which would indirectly cause disturbance of the individual health status (Mallat, 1985; Reddy and Waskale, 2013). In the present study, the most frequent alteration identified in gills of juvenile *Solea* spp. was aneurysm. A high prevalence of this lesion is seemingly linked to pollutant exposure or to environmental stress conditions (Camargo and Martinez, 2007; Costa et al., 2009). However, since baseline gill histopathology data in *Solea* spp. are lacking hitherto, the environmental relevance of the prevalence of aneurysm herein recorded cannot be certainly concluded.

Unlike in the case of hepatic histopathology, higher prevalence and total gill histopathological index were recorded in the latest years of the study period. As gills are the first organ in contact with the environment, this increase in gill histopathological alterations might indicate recent stress conditions such as a new contaminant input or the remobilisation of older xenobiotics contained in the sediment (Costa et al., 2009).

Prevalence of most gonad lesions recorded in juvenile *Solea* spp. from the Bilbao estuary was comparable to levels measured in previous studies on adults from the Basque continental shelf (Cuevas et al., 2015a). Exceptionally, the MMC prevalence was lower in juvenile *Solea* spp. from the Bilbao estuary than in offshore adults. This is conceivable because the prevalence of MMCs is known to increase with the age of fish (Agius and Roberts, 2003). Interestingly atresia was recorded in female gonads of juveniles. Atresia has been described in adult soles from the Basque continental coast (Cuevas et al., 2015b). It is known that atresia occurs naturally prior or during the spawning period (Whitthames and Greer Walker, 1995), but the phenomenon has also been detected in response to unfavourable environmental conditions (Blazer, 2002; Reynolds et al., 2003). Thus, the occurrence of atresia in juveniles should be less common than in adults. Nevertheless, atresia levels decreased throughout the years suggesting an improvement in environmental conditions. On the other hand, it is known that fish populations inhabiting downstream of wastewater treatment plant effluents show alterations in gonad and gamete development such as intersex (Jobling et al., 2002; Woodling et al., 2006; Puy-Azurmendi et al., 2013). Although in a previous study intersex condition was detected in thicklip grey mullet (*Chelon labrosus*) of the Bilbao estuary (Valencia et al., 2017), no intersex cases were found in *Solea* spp. during the 7-year study suggesting low oestrogenic effects.

Gonad alteration prevalence and total gonad histopathological indices were higher in females than in males, which seems to be a general rule in flatfish (Cuevas et al., 2015a; Köhler, 2004). In females, the highest total gonad histopathological index was observed in 2012 and decreased until 2015 in parallel with the decreasing trend observed in hepatic histopathology. In contrast, a significant increase was observed from 2015 to 2017 coinciding with the rise observed in gills histopathological lesions in 2017; thus, reinforcing the idea that the health status of *Solea* spp. was somehow disturbed in the latest study years.

Overall, chemical and histopathological data obtained throughout seven years of campaigns from the Bilbao estuary showed fluctuations with no clear temporal trend nor data correlations. Previous studies from the Bilbao estuary demonstrated the improvement of the ecosystem quality based on longer-term data including physicochemical, geochemical and biological data (Cearreta et al., 2004; García-Barcina et al., 2006; Borja et al., 2016; Pouso et al., 2018a, 2018b). The collection of chemical and biological samples carried out in the present study should be maintained in the future to confirm the recovery of the health status of the ecosystem. The selection of several sampling areas for sediment and fish collection permitted to assess contaminant levels and their effects in the whole estuary. The application of a histopathological approach in sentinel juvenile sole, which live in estuaries until maturation, permitted to spot signs of disturbance of ecosystem health status.

Concluding remarks

The 7-year campaigns carried out in the Bilbao estuary permitted to survey the general health status of the area, a system experiencing a recovery process of its ecological quality from a very much deteriorated situation. Contaminant levels detected in the sediments from the present study suggested that some toxicological adverse effects could still be occurring. The application of histopathological approaches to diagnose the health status of juvenile *Solea* spp. revealed alterations of mild severity fluctuating throughout the years of the study period (2011-2017). The lack of a clear temporal trend in contaminant levels or histopathological approaches can be explained by the high variability that characterises estuarine ecosystems. This drawback can be compensated by the application of long-term monitoring programmes, which would contribute to reliably identify existing temporal trends by combining data on sediment chemistry and the health status of juvenile soles that live in the estuary.

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Chapter II

Biological responses and toxicopathic effects elicited in *Solea senegalensis* juveniles on exposure to contaminated sediments under laboratory conditions

Congress

Briaudeau T., Huerga E., Marigómez I., Garmendia L., Garmendia J.M., Zorita I., Izagirre U. Biomarkers, histopathology and condition indices in sole (*Solea senegalensis*) exposed to contaminated sediments. 10th Iberian and 7th Iberoamerican Congress on Environmental Contamination and Toxicology (CICTA), Vila Real, Portugal, 14-17 July 2015. Platform presentation (T. Briaudeau).

Briaudeau T., Zorita I., Marigómez I., Izagirre U. Lysosomal biomarkers in juvenile *Solea* spp. for early warning assessment of marine ecosystem health. 30th new European Society for Comparative Physiology and Biochemistry (ESCPB), Barcelona, Spain, 4-7 September 2016. Platform presentation (T. Briaudeau).

Briaudeau T., Marigómez I., Izagirre U. Towards a Tissue-Array technology for the assessment of cell and tissue-level biomarkers in marine pollution biomonitoring programmes. 30th new European Society for Comparative Physiology and Biochemistry (ESCPB), Barcelona, Spain, 4-7 September 2016. Poster presentation.

Briaudeau T., Alves Dos Santos A., Guerrero-Limón G., Marigómez I., Zorita I., Izagirre U. Biochemical biomarkers and histopathology in juvenile *Solea senegalensis* for early warning assessment of marine ecosystem health. SETAC Europe 28th annual meeting, Rome, Italy, 13-17 May 2018. Poster presentation.

Abstract

Adverse pollution effects persist in estuaries experiencing ecological recovery after long-term deterioration caused by pollution, as suggested in field studies by the presence of elevated levels of contaminants in the sediments and of disturbed health condition in resident benthic fish. Although the association between the presence of contaminants in sediments and the toxicopathic effects in fish is intricate, it can be elucidated by applying whole-sediment toxicity assays. Presently, *Solea senegalensis* juveniles were exposed under laboratory conditions to contaminated sediments from the Basque Coast (SED1: from the moderately polluted Plentzia Estuary; SED3: from the highly polluted Pasaia Harbour; and SED2: 1:1 v/v mixture of SED1 and SED3), for which physicochemical properties and levels of contaminants were determined. Sole juveniles (n=12 per group) were retrieved after 7 and 28 d exposure. Biometry data were recorded. Liver, brain, gills, and gonads were dissected out and processed to determine biomarkers of oxidative stress and neurotoxicity, lysosomal biomarkers and histopathology. Sediments differed in organic matter content, granulometry and redox potential, as well as in contaminant profile and levels. In the three whole sediments, moderate concentrations of metals were recorded, the highest being found in SED3, and levels of organic chemicals (PAHs, PCBs and pesticides) were low. Overall, biological responses were consistent with levels of contamination reported in source sediments, the most marked toxicopathic effects being detected upon exposure to SED3 and particularly at day 28. Enhanced hepatic GST activity was detected in soles from all experimental groups, demonstrating a toxic effect from all sediments whilst CAT inhibition was most pronounced in SED3 exposed soles. Lysosomal enlargement was initiated in all experimental groups but early lysosomal membrane destabilisation and changes in lysosomal content were more clearly detected in soles exposed to SED3. Histopathological analysis indicated that liver was the most affected target organ and showed higher toxicity from SED3. The IBR/n index (Integrative Biological Response) confirmed that exposure to the three sediments caused significant biological responses and toxicopathic effects of diverse magnitude ($IBR/n_{SED3}>IBR/n_{SED2}>IBR/n_{SED1}$). The present sediment toxicity assay based on chemical analysis of sediments and biomarkers and histopathology in sole juveniles has confirmed the association between the presence of contaminants in sediments and the emergence of toxicopathic effects in sole juveniles.

Résumé

Les effets de la pollution marine persistent dans les estuaires en reconstitution, même après une diminution majeure de la pression anthropique. En effet, de nombreux contaminants sont encore détectés dans les différents compartiments de l'écosystème tels que les sédiments. Néanmoins, l'association entre la présence de contaminants dans les sédiments et les effets toxicopathologiques enregistrés dans le biote peut être complexe. L'application de tests de toxicité utilisant des sédiments naturels permet d'évaluer en laboratoire les effets biologiques potentiels des contaminants contenus dans le substrat. Dans l'étude suivante, des soles juvéniles (*Solea senegalensis*) ont été exposées à des sédiments de la côte basque (SED1: mélange provenant de l'estuaire de Plentzia, moyennement pollué; SED3: du port très pollué de Pasaia Harbour; et SED2: 1:1 v/v, mélange de SED1 et SED3), pour lesquels les propriétés physicochimiques et les niveaux de contaminants ont été déterminés. Des échantillons de foie, cerveau, branchies et gonades ont été prélevés dans chaque groupe expériemental (n=12) après 7 et 28 jours d'exposition aux sédiments afin d'y analyser des biomarqueurs de stress oxidatif, de neurotoxicité et de lysosomes, ainsi que l'histopathologie des branchies, du foie et des gonades. La teneur en matière organique, la granulométrie et le potentiel rédox, ainsi que le profil et les concentrations de contaminants variaient entre sédiments. Globalement, les niveaux de contaminants métalliques étaient modérés dans les trois sédiments, les plus élevés ayant été détectés dans SED3. Les contaminants organiques tels que les HAP, PCB et pesticides, étaient présents à faibles concentrations. Dans l'ensemble, les réponses biologiques concordaient avec les niveaux de contamination de chaque sédiment, les effets toxicopathologiques les plus marqués ayant été enregistrés dans le cas de soles exposées au SED3, en particulier après 28 jours. L'induction enzymatique de la glutathion-*S*-transférase hépatique a révélé des effets toxiques pour les trois sédiments tandis que l'inhibition de la catalase du foie était plus prononcée chez les soles exposées au SED3. Des signes d'altération de structure lysosomale (élargissement lysosomal) ont été détecté dans tous les groupes expérimentaux, mais la déstabilisation de la membrane lysosomale et les changements du contenu lysosomal ont été plus clairement détectées dans les soles exposées au SED3. Selon les analyses histopathologiques, le foie était l'organe le plus touché par la toxicité des sédiments, en particulier dans le cas de SED3. L'intégration des réponses biologiques sous forme de l'index « Integrative Biological Response index » (IBR/n) a confirmé la toxicité de chaque sédiment, à différents niveaux de sévérité (IBR/n_{SED3}>IBR/n_{SED2}>IBR/n_{SED1}). L'analyse chimique des sédiments et l'intégration de biomarqueurs mesurés chez la sole juvénile a permis de confirmer le lien entre la présence de contaminants dans les sédiments et les effets toxicopathologiques détectés chez la sole.

Laburpena

Zelai ikerketetan antzematen diren kutsatzaile maila altuak zein arrain bentonikoen osasun eskasak, agerian usten dute kutsaduraren efektuak iraunkorrak izan daitezkeela epe luzez kaltetuak egon diren estuarioetan.

Sedimentuen dauden kutsatzaileen eta hauek eragin ditzaketen efektu toxikopatikoen arteko erlazioa konplexua den arren, sedimentu gordinekin aplika daitezkeen toxizitate entseguek erlazio horien nondik norakoak argitzeko lagungarriak izan daitezke. Honen harira, ikerlan honetan, *Solea senegalensis* jubenilak euskal kostaldeko sedimentu desberdinetara esposatu ziren laborategi baldintzetan (SED 1: Plentziar estuarioko moderatuki kutsatutako sedimentua; SED3: Pasaia portuko sedimentu oso kutsatuak; eta SED2: SED1 eta SED3 sedimentuen nahastea 1:1 v/v proportzioan). Aurretiaz, sedimentuen propietate fisikokimikoak eta kutsadura mailak aztertu ziren. 7 eta 28 egunetako esposizioa eta gero, mihi-arrain jubenilen (n=12 talde bakoitzeko) biometria datuak erregistratu ziren eta gibel, garun, zakatz eta gonadak disekzionatuak eta prozesatuak izan ziren, estres oxidatibo eta neurotoxizitate biomarkatzaileak, biomarkatzaile lisosomikoak zein, azterketa histopatologikoak burutzeko. Sedimentuek ezberdintasunak ageri zituzten materia organiko kantitatean, granulometrian eta erredox potentzialean; baita, kutsadura perfil eta mailetan ere. Hiru sedimentuetan metal kontzentrazio moderatuak eta altuenak SED3 sedimentuan erregistratu ziren. Aitzitik, produktu kimiko organikoen mailak (HAP, PCB eta pestizidak) baxuak izan ziren. Orokorrean, kutsatzaileekiko erantzun biologikoak bat zetozken neurtutako kutsadura mailekin. Efektu toxikopatiko azpimarragarrienak SED3 sedimenturako esposizioan antzeman ziren, bereziki 28. egunean. GST aktibitate hepatikoaren gorakada eman zen talde esperimental guztietan, aztertutako sedimentu guztien toxikotasun maila minimo bat bermatuz. Aldiz, CAT inhibizioa azpimarragarriagoa izan zen SED3 sedimenturetara esposatutako banakoetan. Hasiera batean, talde esperimental guztietan sistema lisosomikoaen emendapena nabaria izan zen arren, mintz lisosomikoaren desegonkortze goiztiarra eta eduki lisosomikoaren aldaketak bereziki SED3 sedimentura esposatutako arrainetan antzeman ziren. Analisi histopatologikoei begira, gehien kaltetutako organoa gibela izan zen, afekzio/toxikotasun handiena SED3 sedimentuan hauteman zelarik. IBR indizeak (Integrative Biological Response) hiru sedimentuetarako esposizioak eragindako erantzun biologiko esanguratsuak berretsi zituen (IBR/nSED3>IBR/nSED2>IBR/nSED1). Sedimentuen analisi kimikoetan, zein Solea jubeniletan neurtutako erantzun biologikoetan eta histopatologikoetan oinarritzen den entsegu honek, sedimentuetako kutsatzaileen eta Solea jubeniletan antzeman daitezkeen efektu toxikopatikoen arteko asoziazioa berresten du.

1. Introduction

Due to degradation over decades by anthropogenic activities, many estuaries from developed countries have undergone processes of recovery for years; however high levels of contaminants are still detected in their sediments (e.g. in the Bilbao Estuary; Borja et al., 2016; Cajaraville et al., 2016; Chapter 1). Indeed, estuarine sediments can remain contaminated for extended periods even after the cessation or reduction of pollutant sources (Wolanski and Richmond, 2008). Sediments are a complex milieu that can act as a sink for chemical compounds and also as a source of pollutants by releasing previously trapped chemicals (Eggleton and Thomas, 2004); therefore, they may pose a potential ecological risk for the resident biota, as recorded for instance in the case of flatfish, *Solea* spp. (Oliva et al., 2010, 2012a, 2012b, 2013; Fonseca et al., 2011a; Gonçalves et al., 2013; Chapter 1).

In natural sediments, toxicity not only depends on the presence of cocktails of contaminants but also on the different relative concentration, speciation and mobility of each chemical in the cocktail (Chapman, 1990; Eggleton and Thomas, 2004). Thus, toxicity testing using whole (native) sediments represents the widest variety of possible exposure routes for sediment toxicity assessment (Hallare et al., 2011). Toxicity assays using flatfish and other benthic fish have been often applied to assess the complex toxicity of these sediments. These organisms live in direct contact with the sediment and their general health status can be thus directly linked to the presence of pollutants in this environmental compartment (Jiménez-Tenorio et al., 2008; Costa et al., 2009a, b; Vicente-Martorell et al., 2009; Kerambrun et al., 2012). Sole is a common flatfish inhabiting along the Atlantic Iberian coast (Lagardere et al., 1979; Quéro et al., 1986; Quéro and Vayne, 1997) where it is subject to intensive farming (Fuchs, 1981; Dinis et al., 1999; Imsland et al., 2003). This provides environmentalists with an invaluable opportunity for its use as test organism for sediment toxicity assays.

The biological effects of pollutants in sole have been investigated in laboratory experiments using early life stages (larvae and fries) and juveniles exposed both to waterborne pollutants and to whole-sediment (Claireaux et al., 2004; Riba et al., 2004; Jiménez-Tenorio et al., 2008; Salamanca et al., 2008; Costa et al., 2009a, b; Ribecco et al., 2012; Martins et al., 2015). Yet, whereas 2-3 yr old sole juveniles are commonly selected in field studies as sentinels representative for estuaries (Oliva et al., 2010, 2012a, 2012b, 2013, 2014; Gonçalves et al., 2013; Sànchez-Nogué et al., 2013; Cuevas et al., 2015b; Chapter 1), whole-sediment toxicity assays with juveniles of that age class are rare (Jiménez-Tenorio et al., 2008). Indeed, assays are usually conducted using small (young) juveniles or fries (Riba et al., 2004; Costa et al., 2009a, b; Ribecco et al., 2012; Martins et al., 2015). The transfer of toxicity results from laboratory assays to risk assessment in estuaries demands more research studies dealing with whole-sediment toxicity assessment in 2-3 yr old sole juveniles.

The toxicity of chemicals in mixtures (e.g. contaminated sediments) can be assessed based on several biological endpoints to demonstrate exposure to, bioavailability and

effects of chemicals. Determining biological responses at different levels of biological complexity is critical to assess pollutant toxicity in an integrative way (Davies and Vethaak, 2012; OSPAR Commission, 2013). Biochemical, cell and tissue-level biomarkers are considered early warning biological responses to exposure to chemicals, among other stressors, and are indicators of the general health status of the biota (UNEP/RAMOGE, 1999).

Oxidative stress, reported in liver of fish exposed to chemical pollutants (Regoli et al., 2011; Alijani et al., 2017), can be reflected in enhanced production of reactive oxygen species (ROS), disturbance of antioxidant defences and alterations in xenobiotic metabolism (Di Giulio et al., 1989; Livingstone, 2001; Roméo et al., 2013; Regoli and Giuliani, 2014). Particularly, changes in certain antioxidant enzyme activities, such as superoxide dismutase (SOD) and catalase (CAT), are considered early warning biomarkers indicative of disturbance of antioxidant defences in aquatic animals (Valavanidis et al., 2006; Kroon et al., 2017). In sole, the different levels of SOD and CAT activities recorded in the field are seemingly related to the presence of pollutants in the sediment (Oliva et al., 2010, 2012b; Fonseca et al., 2011a, 2011b; Gonçalves et al., 2013; Jebali et al., 2013; Siscar et al., 2015; Kroon et al., 2017; Ghribi et al., 2019). Alas, antioxidant responses in sole in the laboratory have been investigated only on exposure to waterborne chemicals (Solé et al., 2008; López-Galindo et al., 2010a, 2010b) and hence whole-sediment toxicity assays are needed.

Xenobiotic metabolism in fish involves the action of several enzymes, amongst which the glutathione-*S*-transferase (GST) is responsible for the biotransformation of chemicals and their conjugation by reduced glutathione (van der Oost et al., 2003; Kroon et al., 2017). Particularly, enhanced GST activity was reported in sole exposed to diverse chemicals both in the field and in waterborne toxicity assays (Fonseca et al., 2011a; 2011b; Salamanca et al., 2008; Díaz-Garduño et al., 2018); yet, whole sediment toxicity assays are rare (Jiménez-Tenorio et al., 2008; Ghribi et al., 2019).

Neurotoxicity can be exerted on aquatic organisms by many contaminants found in the sediment (Legradi et al., 2018), affecting their behaviour (swimming activity, visual or breathing impairments) and survival (Massei et al., 2019). A neurotoxicity biomarker frequently used in fish is brain acetylcholinesterase (AChE) inhibition (Grue et al., 1997; Heath et al., 1997; Minier et al., 2000; Davies and Vethaak, 2012; Burgeot et al., 2017), which has been also investigated in sole (López-Galindo et al., 2010a, b; Oliva et al., 2012a; Solé et al., 2012; Jebali et al., 2013; Siscar et al., 2013; Ghribi et al., 2019).

Lysosomal alterations are considered precursors of early histopathological lesions (Köhler et al., 1992, 2002) and hence they are used as early-warning biomarkers of the general health status of marine organisms subject to a variety of environmental stressors (UNEP/RAMOGE, 1999; JAMP, 2003; ICES, 2006; Davies and Vethaak, 2012). They have been commonly reported in fish hepatocytes as non-specific toxic responses (Hinton et al., 2001; van der Oost et al., 2003; Au, 2004). Particularly, lysosomal enlargement, membrane destabilisation and changes in lysosomal content have been described in wild

flatfish from polluted sites (Broeg et al., 2002, 2005; Einsporn et al., 2005; Baršienė et al., 2006; Burgeot et al., 2017). Moreover, these lysosomal responses have been related with sediment pollution (Baršienė et al., 2006; Burgeot et al., 2017).

Histopathology can provide valuable data to assess the general biological effects of contaminants in fish, including when these occur in mixtures and complex environments, like it happens in the case of sediments (Bernet et al., 1999; Gonçalves et al., 2013; Feist et al., 2015). Moreover, histopathological lesions represent medium-term responses to pollutants that can be used to link molecular and cellular responses to their ecological consequences e.g. at population level (Hinton and Lauren, 1990; Au, 2004; Lang et al., 2006). In fish, the target tissues most commonly used for histopathological diagnosis are gills, liver and gonad (Arellano et al., 1999; Hinton et al., 2001; Costa and Costa, 2008; Jiménez-Tenorio et al., 2008; Costa et al., 2009b; 2011; Lujić et al., 2013; Feist et al., 2015). Additionally, gonad examination at the microscope is frequently used to identify the gender and reproductive status of the fish (Blazer, 2002; Solé et al., 2016).

Overall, histopathological lesions in fish can be classified into four categories (Takashima and Hibiya, 1995; Bernet et al., 1999): (1) circulatory disturbances; (2) inflammatory responses; (3) regressive changes; and (4) progressive changes. Circulatory disturbances inform on the condition of blood and fluid flow (Bernet et al., 1999). For instance, haemorrhage has been reported in the liver in sole exposed to contaminated sediments (Costa et al., 2011). Inflammatory responses are interpreted as indicators of disturbance of the general health status as a result of age, infectious disease or exposure to chemicals (Zorita and Cuevas, 2014). Thus, enhanced occurrence of melanomacrophage centers in liver is considered a sign of environmental deterioration due to pollution or other causes (Manera et al., 2000). Regressive changes lead to reduction or loss in the function of the affected organ. In gills, epithelial lifting increases the distance between blood vessels and the milieu, hampering gas exchange and thus interfering with respiratory and osmoregulatory functions (Reddy and Waskale Kusum, 2013). Necrotic foci are degenerative lesions characterised by an irreversible loss of cellular integrity and have been identified in sole liver in response to contaminated sediments bearing cocktails of xenobiotics (Costa et al., 2011). Oocyte atresia is considered a regressive change that occurs during the normal reproductive cycle in fish but has been also identified in response to environmental stressors (Witthames et al., 1995; Chapter 1). Progressive changes are the consequence of an abnormally enhanced activity of cells leading to tissue hypertrophy (cell enlargement) or hyperplasia (cell proliferation) (Bernet et al., 1999); which can ultimately produce organ malfunction through e.g. fusion of secondary lamellae in gills (López-Galindo et al., 2010a; Movahedinia et al., 2012). Upon examination of lesions included in the aforementioned four categories, results can be integrated into indices of the biological effects exerted by pollutants (Bernet et al., 1999; Lang et al., 2006; Costa et al., 2009b, 2011; Cuevas et al., 2015a, 2015b; Ghribi et al., 2019; Chapter 1).

In addition, biomarkers recorded at different levels of biological complexity and based on diverse biological endpoints and technological approaches (biomarkers of oxidative stress, biotransformation, neurotoxicity and general stress, and histopathology) can be integrated into indices of health disturbance (Broeg et al., 2005; Marigómez et al., 2013). This integration is helpful to understand the effects of pollution when chemicals occur in mixtures in the complexity of the field and to support environmental managers in risk assessment and monitoring practice. Within this framework, the Integrative Biological Response (IBR) index has been previously applied in fish and other marine organisms based on a variety of combinations of biomarkers (Broeg and Lehtonen, 2006; Brooks et al., 2011; Cravo et al., 2012; Serafim et al., 2012; Marigómez et al., 2013; Rementeria et al., 2016).

The current investigation aimed at relating the occurrence of contaminants mixture in sediments with biological responses elicited in *Solea senegalensis* juveniles upon exposure to whole sediments under controlled laboratory conditions for 28 d. Biomarkers of oxidative stress and neurotoxicity, lysosomal biomarkers and histopathology were determined after 7 and 28 d exposure and integrated as IBR/n index.

2. Material and methods

2.1. Experimental setup

Sediments used for the present study were collected from two estuaries with different levels of pollution along the Basque coast, Bay of Biscay (Figure 1). The first sampling site was located in the Plentzia estuary (43° 25' N, 2° 57' W). Sediment was collected nearby the village of Plentzia. The area is considered relatively clean with no significant industrial activity and low levels of PAHs and PCBs (Saiz-Salinas, 1997; Borja et al., 2006; Orbea and Cajaraville, 2006; Cortada and Collins, 2013). It is worth mentioning that the population in this area increases significantly during summer. The second sampling site was situated in the Pasaia harbour (43º 20' N, 01º 56' W), which is subject to relevant industrial activities, dredging operations, and hydrometallurgical and mining related discharges (Belzunce et al., 2004; Tueros et al., 2009; Montero et al., 2013). Sediments used for the present study were collected using shovels in Plentzia and a Day grab in Pasaia. In both cases, the sediments were transported to the laboratory, where they were homogenised and preserved at 4ºC until use. A third sediment was prepared in the laboratory by gently mixing (1:1 v/v) the two source sediments. Experimental sediments are referred to as SED1 (Plentzia), SED2 (mixture) and SED3 (Pasaia). Prior to the exposure experiment (48 h) each sediment was placed at the bottom (8 cm layer) of a 500 L-capacity polypropylene tank with constant aeration and water flow. Water circulation (1.2 l/min) was equivalent to four water renewals per day and was so set to minimise sediment loss.

Solea senegalensis juveniles (22.2±2.2 cm standard length; 112.5±31.7 g total wet-wt) were exposed to the three experimental sediments (SED1, SED2 and SED3) for 28 d at optimal stocking density (4-6 kg/m^2; Schram et al., 2006). Water parameters were

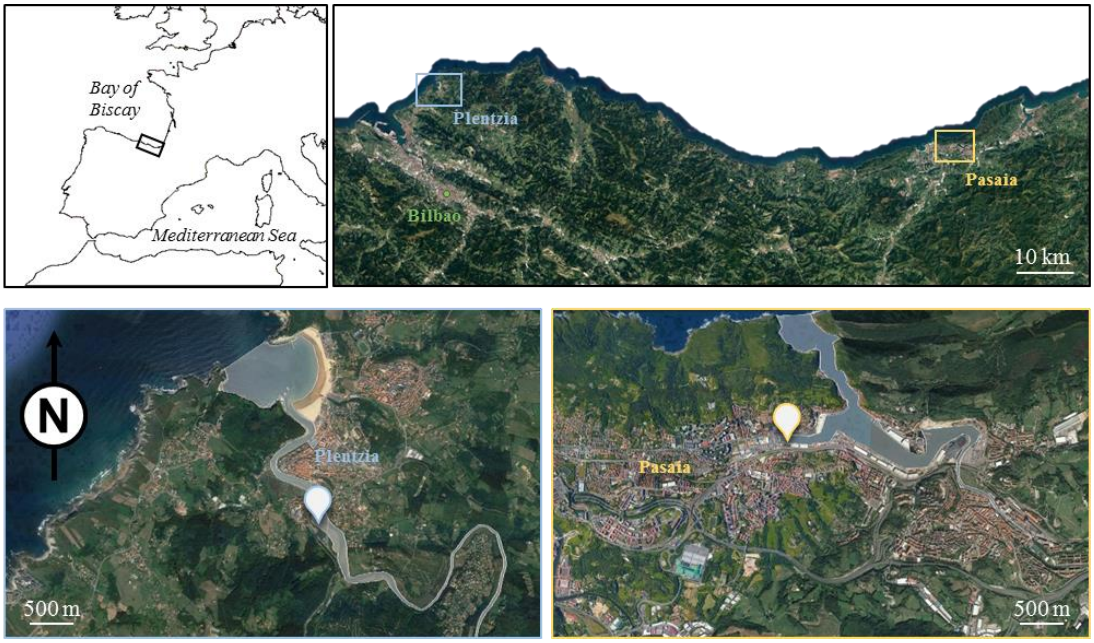


Figure 1. Map of the Basque Coast showing the sediment sampling sites in the Plentzia estuary (left) used to produce SED1 and the Pasaia harbour (right) used to produce SED3.

monitored daily to ensure optimal conditions: pH=8, salinity=31-33 PSU, temperature=19-20ºC, dissolved O_2=6-8 mg/l and total ammonia=0 mg/l. Photoperiod throughout the experiment was set at 12:12 h light:dark. Fish were daily fed with commercial food (0.3 g/fish; BioMar Iberia S.A., Dueñas, Spain).

2.2. Physicochemical and chemical analyses of sediments and water

Samples of experimental sediments were collected from each experimental group at days 3 and 28.

Sediment granulometry was determined after running dried sediment samples (60ºC, 24 h) through a column of sieves according to Holme and McIntyre (1971). The proportion (%) of gravel (>2 mm), sand (2 mm-63 µm) and mud (<63 µm) was calculated and particle size distribution was interpreted using the GRADISTAT software (Blott and Pye, 2001). The organic matter content was determined as weight loss (%) on ignition at 450ºC for 5 h (Dean, 1974). The redox potential was determined by using a silver combined electrode (Orion 977800) connected to a pH/mV meter (Orion 710A).

Metals in sediments (Cd, Cr, Cu, Hg, Ni, Pb and Zn) were measured in acid extracts from the sediment fine fraction (<63 µm). Briefly, dried sediment was digested in an acid mixture (2HCl:1HNO_3) using a microwave system (MARS 5 Xpress CEM Corporation Instrument). Afterwards, metal levels were determined by Atomic Absorption Spectrometry (AAS; AAS800 Perkin Elmer). Cadmium was analysed by Transversely Heated Graphite Atomizer (THGA) graphite furnace, using Zeeman background correction (Katskov et al., 1998). Chromium, Cu, Ni, Pb and Zn were determined in an

air acetylene flame (Allan, 1962). Total Hg was measured by quartz furnace AAS following cold vapour method (Deng et al., 2009). Analytical accuracy was checked by the PACS-2 reference material (National Research Council of Canada, CNRC) and the measured values were found to be within the certified range (Larreta et al., 2012).

Polycyclic aromatic hydrocarbons (PAHs; 18 in total) in sediments were measured in the bulk fraction (<2 mm). Samples (5-10 g) were pre-concentrated with a mixture of solvents (pentane:dicloromethane; 50:50) by accelerated solvent extraction (Dionex ASE200 System). Organic extract was purified by Gel Permeation Chromatography (GPC). Sulphuric acid (8 ml) was added to PAH extract prior to centrifugation. Organic phases were collected and determined by Gas Chromatography-Mass Spectrometry (GC-MS) using Agilent 6890 GC coupled with Agilent 5973 MSD.

Contaminant levels were compared with the values of the Effect Range Low (ERL) and Effect Range Median (ERM) for metals, PCBs and PAHs (Long et al., 1995). The contaminant potential to cause adverse biological effects was assessed through the estimation of Sediment Quality Guideline Quotients (SQG-Qs). The SQG-Qs were calculated as the ratio between the content of individual chemicals and their respective ERM value. The sediments were then ranked as proposed by MacDonald et al. (2004) according to their toxicological risk for each class of contaminants (metals, PCBs and PAHs): SQG-Q < 0.1 as non-impacted sediments; 0.1-1 as moderately impacted and > 1 as strongly impacted. In this approach, the mean of individual SQG-Qs of each chemical group was calculated.

2.3. Fish biometry

Individual wet-wt (W in g) and length (L in cm) and liver and gonad wet-wt (LW and GW in g, respectively) were recorded to calculate (a) $K=W\times100/L^3$; (b) HSI= LW×100/W; and (c) GSI=GW×100/W; where K is the condition factor, HSI is the hepatosomatic index, and GSI is the gonadosomatic index.

2.4. Analysis of contaminants in liver

Due to the high fat content of the liver of sole, only metals concentrations could be reliably measured. For this purpose, liver tissue of 6 fish per treatment were pooled to obtain a minimum of 1 g dw per group. After lyophilisation, tissue samples were digested in an acid mixture (2HCl:1HNO_3) using a microwave system (MARS 5 Xpress CEM Corporation Instrument). Metal content (Cr, Mn, Fe, Ni, Zn, Cd, Hg and Pb) was determined by Inductively Coupled Plasma - Mass Spectrometry (ICP-MS; Bartolomé et al., 2010; Navarro et al., 2010). Copper concentration was analysed by Atomic Emission Spectroscopy (ICP-AES).

2.5. Biochemical determination of enzyme activities

At days 7 and 28 of exposure, liver and brain samples were dissected out, rapidly frozen and maintained at -80ºC until use. Samples were processed for biochemical analysis; they

were homogenised (1:4 for liver and 1:5 for brain) in 0.1 M potassium phosphate buffer (pH 7.4) and centrifuged for 30 min at 12000 *g* at 4ºC to obtain the post-mitochondrial supernatant (PMS). Catalase (CAT), superoxide dismutase (SOD) and glutathione-*S*-transferase (GST) enzyme activities were determined in liver PMS and acetylcholinesterase (AChE) activity in brain PMS using a BioTek Eon microplate spectrophotometer. Enzyme activities were expressed as a function of the protein concentration in the samples. Total protein content in the homogenates was measured in triplicate at 595 nm following Bradford's method adapted to microplate and using bovine serum albumin as standard (Bradford, 1976; Guilhermino et al., 1996). All enzyme assays were performed at 25ºC.

Catalase (CAT). CAT activity was determined by the method of Claiborne (1985) by measuring the rate of enzyme decomposition of hydrogen peroxide (H_2O_2) determined as absorbance decrease at 240 nm. The reaction medium (final volume of 10 ml) contained 9977 µl of 50 mM phosphate buffer (pH 7.0) and 23 µl of hydrogen peroxide (H_2O_2; 30% v/v). The reaction was started by the addition of 5 µl of samples to 295 µl of reaction medium. Absorbance decrement was measured for 3 min at 240 nm. Results were expressed as µmol H_2O_2/min/mg protein.

Superoxide dismutase (SOD). SOD activity was determined by a colorimetric method using a SIGMA kit (SOD Determination kit; ref: SIGMA 19160) to measure the superoxide anion reduction as proportional to the SOD inhibition activity. Each well contained 200 µl of WST (water soluble tetrazolium salt) working solution, 20 µl of enzyme working solution and 20 µl of sample and were left incubating at 37ºC for 20 min. Three different blanks were prepared for the assay: Blank 1 (200 µl of WST working solution, 20 µl of enzyme working solution and 20 µl of ultrapure water); Blank 2 (200 µl of WST working solution, 20 µl of dilution buffer and 20 µl of sample); and Blank3 (200 µl of WST working solution, 20 µl of dilution buffer and 20 µl of ultrapure water). Absorbance was measured at 450 nm and SOD activity (inhibition rate %) was calculated as follow:

$$\text{SOD activity (inhibition rate \%)} = \frac{(A1-A3)-(AS-A2)}{(A1-A3)} \times 100;$$

where A1 is the absorbance of Blank 1, A2 is the absorbance of Blank 2, A3 is the absorbance of Blank 3 and AS is the absorbance of the samples.

Glutathione-S-transferase (GST). GST activity was determined by the Habig's method (Habig et al., 1974) adapted to microplate and using bovine serum albumin as standard (Guilhermino et al., 1996). Enzyme activity was measured following the formation of thioether by conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB). The reaction medium contained 9.9 ml of 100 mM potassium phosphate buffer (pH 7.4), 1.8 ml of 10 mM GSH solution and 300 µl of 60 mM CDNB solution. Each well contained 100 µl of samples and 200 µl of reaction medium. Enzyme activity was measured at 340 nm for 6 min and expressed as nmol/min/mg protein.

Acetylcholinesterase (AChE). AChE activity was determined according to the Ellman 's colorimetric method of (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996) by measuring the formation of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) at 412 nm. The reaction medium contained 200 µl of 75 mM acetylcholine solution, 1 ml of 10 mM DTNB and 30 ml of 100 mM potassium phosphate buffer (pH 7.4). Each well contained 50 µl of samples and 250 µl of reaction medium. After 10 min of incubation, enzyme activity was recorded over 10 min. AChE activity was expressed as specific activity (nmol DTNB/min/mg protein).

2.6. Lysosomal biomarkers

At days 7 and 28 of exposure, liver samples were dissected out, rapidly frozen and maintained at -80ºC until use. Frozen samples were processed using Tissue Array (TA) technology (Array Mold® Kit; n°20015-A) and TA blocks were cut at -27ºC using a Leica CM 3050S cryotome.

Lysosomal membrane stability (LMS). The determination of lysosomal membrane stability was based on the time of acid labilisation treatment required to produce the maximum staining intensity according to UNEP/RAMOGE (1999), after demonstration of acid phosphatase (AcP) activity in hepatocyte lysosomes. Ten serial cryotome sections (10 µm) were subject to acid labilisation in intervals of 0, 2, 4, 6, 8, 10, 15, 20, 25, 30, 40 and 50 min in 0.1 M citrate buffer (pH 4.5, containing 2.5% NaCl) in a shaking water bath at 37ºC. The demonstration of AcP activity was performed by incubation of the sections in a substrate incubation medium (naphthol AS-BI-phosphate, dimethylsulfoxide, 0.1 M citrate buffer at pH 4.5, containing 2.5% NaCl and low viscosity polypeptide, Polypep®) for 20 min at 37ºC, in a shaking bath. Rinsed sections (3% NaCl at 37ºC for 5 min) were stained at room temperature with diazonium dye Fast Violet B salt (1 mg/ml in 0.1 M phosphate buffer, pH 7.4) for 9 min. Slides were fixed in Baker's formol calcium containing 2.5% NaCl for 10 min at 4ºC, rinsed in distilled water and mounted in Kaiser´s glycerine gelatine.

The time of acid labilisation treatment required to produce the maximum staining intensity was assessed under a light microscope as the maximal accumulation of reaction product associated with lysosomes (UNEP/RAMOGE, 1999) and was denoted as the Labilisation Period (LP; in min). Four determinations were made per individual; for each area, the first maximum staining peak was considered to determine the LP value (ICES, 2015). A final LP value was calculated for each individual fish as the mean of the four LP values determined in each area.

Lysosomal Structural Changes (LSC). The determination of changes in the size and numbers of lysosomes was made according to the method described by Cajaraville et al. (1989) for mussels, further on adapted to fish liver by Alvarado et al. (2005), after histochemical demonstration of β-glucuronidase activity in fish hepatocytes. Cryotome sections (8 µm) were incubated in freshly prepared β-glucuronidase substrate incubation medium (naphthol AS-BI-β-glucuronidep, 50 mM sodium bicarbonate, 0.1 M acetate

buffer at pH 4.5, containing 2.5% NaCl and polyvinyl alcohol at 15%) for 20 min at 37ºC. Slides were rinsed (2.5% NaCl at 37ºC for 2 min) and transferred to a postcoupling medium (Fast Garnet, 0.1 M phosphate buffer at pH 7.4 containing 2.5% NaCl) for 10 min at room temperature, in the dark. Sections were fixed in Baker's formol calcium solution containing 2.5% NaCl for 10 min at 4 ºC, rinsed in distilled water and mounted Kaiser's glycerol gelatine.

The structure of lysosomes was assessed through a stereological procedure based on image analysis (BMS, Sevisan) according to Cajaraville et al. (1991). Five measurements using a 100× objective lens were made per individual. The mean value of the following stereological parameters was determined for the lysosomes of each liver sample (Lowe et al., 1981): volume density ($Vv_L=V_L/V_C$), surface density ($Sv_L=S_L/V_C$), surface-to-volume ratio ($S/V_L=S_L/V_L$) and numerical density ($Nv_L=N_L/V_C$); where V=volume, S=surface, N=number, L=lysosomes and C=liver cytoplasm.

Intracellular accumulation of neutral lipids. Changes in levels of neutral lipids were determined according to Marigómez and Baybay-Villacorta (2003), after Oil Red O (ORO) staining to visualise neutral lipids (Culling, 1974). Cryotome sections (8 µm) were fixed in Baker's formol calcium containing 2.5% NaCl for 15 min at 4ºC. Air dried sections were washed in isopropanol (60%) and stained with ORO for 20 min. The staining solution (stable for 1-2 h) was freshly made from a saturated stock ORO solution (0.3% in isopropanol) and kept protected from the light. Stained sections were differentiated in 60% isopropanol, rinsed in water, counterstained with 1% Fast Green FCF for 20 min and mounted in Kaiser's glycerine.

Five measurements using a 40× objective lens were made per individual. The mean value of the volume density ($Vv_{NL}=V_{NL}/V_C$) of neutral lipids was determined; where V=volume, NL=neutral lipids and C=liver cytoplasm.

2.7. Histological processing and histopathological examination

At days 7 and 28 of exposure, gill, liver and gonad samples were dissected out (n=12 per experimental group). Gills were fixed in Bouin's solution for 24 h at 4ºC and rinsed in formic acid (8% v/v) for 24 h at room temperature. Liver and gonad samples were fixed in 4% neutral buffered formol for 24 h at 4ºC. Fixed samples were dehydrated in a graded series of ethanol, cleared and embedded in paraffin (Leica ASP 300S). A minimum of two sections (5 µm) per sample were obtained using a rotary microtome (Leica RM 2125RTS) and were stained with hematoxylin-eosin (H&E; Martoja and Martoja-Pierson, 1970).

Histopathological examination. The examination of histological samples was made under a light microscope (Nikon Eclipse E200) starting with a 4× objective lens for a general description of the organs. Higher power objective lenses (10×, 20×, 40× and 100×) were used for the identification of histopathological lesions.

Hepatic samples were analysed for histopathology based on the recommendations provided by ICES (1997) and the Biological Effects Quality Assurance in Monitoring Programmes (BEQUALM, 2001). The publications by Costa et al. (2009b) on liver and gill histopathology of wild sole juveniles, Zorita and Cuevas (2014) on hepatic lesions commonly recorded in sole and Blazer (2002) on gonad histopathological lesions were used as guidelines to identify lesions in the particular case of sole. Amongst lesions identified, only persistent cases were considered for the calculation of histopathological indices and point alterations were discounted.

Histopathological indices. The semi-quantitative histopathological approach in liver was performed following two different methods.

The first approach was based on the scoring system proposed by Lang et al. (2006) for hepatic histopathology. For this purpose, hepatic lesions were classified into five categories as presented by Feist et al. (2004): (1) non-specific lesions; (2) early non-neoplastic toxicopathic lesions; (3) foci of cellular alteration (FCA); (4) benign neoplasms; and (5) malignant neoplasms. The stage (S) of each lesions recorded was determined as mild, medium and severe, depending on the size of the tissue area affected in the sections and the degree of cellular change observed. Lang's scoring system consisting of 15 lesion scores was used for the assessment of spatial and temporal variation in the lesions recorded. Lesions' scores were determined based on the lesion category and the lesion stage (S). If more than one lesion category was recorded in one specimen, the highest lesion score was used for assessment purposes. From the individual scores, mean histopathology liver lesion scores (LS_{liver}) were calculated for each sampling station and time.

The second semi-quantitative approach was based on the weighted histopathological index developed by Bernet et al. (1999). Accordingly, hepatic lesions were classified into five categories based on their reaction pattern: (1) circulatory disturbances; (2) inflammatory responses; (3) regressive changes; (4) progressive changes; and (5) tumours (neoplams). Each alteration was assigned an importance factor (w) as: (1) minimal pathological importance (the lesion is reversible after the cessation of pollutant exposure); (2) moderate pathological importance (the lesion is reversible in most cases if the exposure ends); (3) marked pathological importance (the lesion is generally irreversible and may lead to partial or total loss of organ function). The stage (a) of each lesion identified was ranked in 4 categories (0, 2, 4 and 6) according to the level of dissemination of the alteration in the organ; where 0 is absence and 6 is high degree of dissemination depending on the size of the tissue area affected in the sections and the degree of cellular change observed. Different histopathological indices were calculated using the lesion importance factor (w) and the lesion stage (a):

- the organ index $I_{org.}$ was calculated for each individual and for each organ as follow:

$$I_{org} = \Sigma_{rp} \Sigma_{alt} (w_{org\ rp\ alt} \times a_{org\ rp\ alt})$$

- the reaction index of an organ $I_{org\ rp}$ was calculated for each individual, each organ and each lesion category:

$$I_{org\ rp} = \Sigma_{alt}\ (w_{org\ rp\ alt} \times a_{org\ rp\ alt})$$

Gills and gonads lesions were also classified according to Bernet et al. (1999) in order to calculate the total histopathological indices integrating the histopathological results from the three organs:

- the total index I_{tot} was calculated for each individual, for all organs:

$$I_{tot} = \Sigma_{org}\ \Sigma_{rp}\ \Sigma_{alt}\ (w_{org\ rp\ alt} \times a_{org\ rp\ alt})$$

- the total reaction index I_{rp} was calculated for each individual and each lesion category, for all organs:

$$I_{rp} = \Sigma_{org}\ \Sigma_{alt}\ (w_{org\ rp\ alt} \times a_{org\ rp\ alt})$$

Histopathological indices. The prevalence of each histopathological alteration was determined as the percentage occurrence of an alteration within each experimental group for gills and liver and within each combination of experimental group and gender for gonads.

Characterisation of the reproductive cycle. Gonad histological sections were analysed at a light microscope for gender and gamete developmental stages determination. Male gamete developmental stages were determined according to García-López et al. (2006) and were classified in five stages as follow: Stage I (early spermatogenesis); Stage II (mid spermatogenesis); Stage III (late spermatogenesis); Stage IV (mature); Stage V (recovery). The identification of gamete developmental stages for females was mainly based on Murua and Motos (2006). Stages were classified as followed: Stage I (growth); Stage II (early vitellogenesis); Stage III (late vitellogenesis); Stage IV (maturation).

2.8. Integrative Biological Response (IBR/n) index

The IBR index (Beliaeff and Burgeot, 2002) was calculated based on the integration of biochemical (GST, CAT), histochemical (Vv_L, LP) biomarkers and hepatic histopathology (I_{liver}) following the calculation method described by Marigómez et al. (2013). The calculation method is based on relative differences between the biomarkers in each given data set. Thus, the IBR index is computed by summing-up triangular starplot areas (multivariate graphic method) for each two neighbouring biomarkers in a given data set, according to the following procedure (Beliaeff and Burgeot, 2002; Devin et al., 2014): (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'=standardized 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 standardized value obtained for each sample to the absolute standardized value of the minimum value in the data set: $y_i = x_i' + |x_{min}'|$; (4) calculation of the Star Plot triangular areas as $A_i = (y_i \times y_{i+1} \times \sin\alpha)/2$, where y_i and y_{i+1} are the standardized values of each biomarker and its next biomarker in the star plot, respectively,

and α is the angle (in radians) formed by each two consecutive axis where the biomarkers are represented in the Start Plot ($\alpha=2\pi/n$; where n is the number of biomarkers); and (5) calculation of the IBR index which is the summing-up of all the Star Plot triangular areas ($IBR=\Sigma A_i$).Since the IBR value is directly dependent on the number of biomarkers in the data set, the obtained IBR value must be divided by the number of biomarkers used (IBR/n; Broeg and Lehtonen, 2006).

In the present work, five biomarkers were integrated for the calculation of the index as IBR/n. Parameters were selected to represent effects of estuarine sediments at different biological organisation levels where biochemical and histochemical parameters demonstrate sub-cellular effects of contaminants and liver histopathology indicate subsequent tissue-level effects.

2.9. Statistical analysis

Statistical analyses were carried out using IBM SPSS Statistics Base 22.0. Homogeneity of variance (Levene´s test) and normality of data (Shapiro's test) were tested before statistical analysis. Two-way ANOVAs were performed to analyse the effects of contaminant, time of exposure and their combination on biomarkers and histopathology. Logarithmic transformation was applied to non-parametric variables (CAT, GST, LP, Vv_L, S/V_L, Nv_L and Vv_{NL}). For normal data, differences between experimental groups and throughout exposure time were tested using the parametric one-way Anova test and the T Student test, respectively. For non-normal data set, the non-parametric Kruskal-Wallis test and Mann-Whithney U test were used to analyse differences in biological data between experimental groups and throughout exposure time. The z-score test and the Pearson's Chi test were used to determine significant differences in histopathological lesion prevalence between experimental groups and throughout exposure time. Significant differences in chemical data were tested with the z-score test. The non-parametric Spearman's rank-order (R) test was used to assess correlations between metal contaminants detected in sediment and liver samples. Level of significance for all analyses was p =0.05.

Table 1. Physicochemical profiles of untreated sediments sediments and experimental group at day 3 and 28: redox potential value (mV), organic matter content (OM, %) and granulometry (gravel >2 mm, sand >63 µm, mud <63 µm, %). Asterisks indicate significant differences between untreated and experimental sediments (z score, $p<0.05$); different letters indicate significant differences between experimental groups (z score, $p<0.05$).

	Untreated sediments		**Experimental sediments**					
			Day 3			**Day 28**		
	SED1	**SED3**	**SED1**	**SED2**	**SED3**	**SED1**	**SED2**	**SED3**
Redox	208	-168.2	368[a]	318[a]	92[b]	106	33	-49
OM	2.2	3	2.9[a]	3.6[b]	3.7[b]	1.8[a]	3.7[b]	4.0[b]
Mud	6.8	7.1*	43.2[a]	68.0[b]	64.8[b]	62.5	65.9	67.2
Sand	89.9	89.2*	56.5[a]	31.7[b]	35.1[b]	37.4	34.0	32.7
Gravel	3.3*	3.7*	0.2[a]	0.2[a]	0[b]	0	0	0

3. Results

3.1. Sediment physicochemical properties and contamination

Untreated SED3 had a negative redox potential that transiently became positive after 3 d experimentation with water renewal under laboratory conditions; SED2 and SED1, however, had always a positive redox potential (Table 1). OM did not differ between untreated and experimental sediments and was higher in experimental SED3 and SED2 than in SED1 (Table 1). Regarding granulometry, it is worth noting that both SED1 and SED3 lost gravel and sand in experimental conditions, more markedly in SED3 (Table 1). Thus, although granulometry was originally similar between untreated sediments, the sand fraction was higher in experimental SED1 than in SED2 and SED3 and the mud fraction was lower (Table 1). However, at day 28 granulometry was similar in the three experimental sediments (Table 1).

The concentrations of Cd, Fe, Hg, Mn, Ni and Pb were comparable in the two source sediments; however, the concentration of Cr, Cu and Zn were higher in SED3 than in SED1 (Larreta et al., 2012). After 3 d experimentation, the concentration of metals raised in both sediments, more markedly in SED3 (e.g., regarding Cd, Cu, Mn, Pb, Zn and the total sum of metals), with the exception of Hg, which was higher in SED1 than in SED3; and remained as such until day 28 (Table 2). However, the concentration of Cr, Fe, Mn and Ni was lower in both sediments after 3 d exposure conditions than in untreated sediments; in contrast, Cd, Cu and Hg concentrations were higher (Table 2).

Table 2. Metals concentrations (mg/kg dw) determined in the fine fraction (<63 µm) of untreated sediments and experimental sediments from days 3 and 28. ∑Metals: sum of Cr, Ni, Cu, Zn, Cd, Hg and Pb. Asterisks indicate significant differences between untreated and experimental sediments (z score, $p<0.05$); different letters indicate significant differences between experimental groups of a same sampling size (z score,

	Untreated sediments		**Experimental sediments**					
			Day3			**Day28**		
	SED1	**SED3**	**SED1**	**SED2**	**SED3**	**SED1**	**SED2**	**SED3**
Cd	0.18	0.43	0.70[a]	0.77[a]	1.37[b]	0.59 [a]	1.02 [b]	0.99 [b]
Cr	21.1*	33.2*	12.7	13.6	16.1	12.3	15.7	14.7
Cu	20.4	47.4*	61.4	80.6	104.9	51.5	89.6	109.0
Fe	17775*	17921*	11863	10786	11380	13017	14084	11885
Hg	0.13	0.1*	0.99[a]	0.89[a]	0.47[b]	0.77 [a]	0.81 [a]	0.50 [b]
Mn	236*	358*	64.5	68.9	84.7	60.8 [a]	76.0 [b]	79.0 [b]
Ni	15.4*	28.2*	11.8	16.2	21.8	12.0	18.7	22.3
Pb	24.2	30.7	46.8[a]	47.7[a]	59.5[b]	37.3	52.7	48.2
Zn	108	213	99.4	122.1	156.3	88.1 [a]	134.4 [b]	141.0 [b]
∑Metals	189.41	353.03	233.75	281.92	360.45	202.56[a]	312.93 [b]	336.69 [b]

Table 3. Toxicological significance (SQG-Qs) from untreated sediments and experimental sediments from days 3 and 28. Effects Range Low (ERL) and Effects Range Median (ERM) calculated by Long et al. (1995). ∑Metals: sum of Cr, Ni, Cu, Zn, Cd, Hg and Pb. LMW PAHs: low molecular weight PAHs; HMW PAHs: high molecular weight PAHs.

	Untreated sediments		**Experimental sediments**							
			Day3			**Day28**				
	SED1	**SED3**	**SED1**	**SED2**	**SED3**	**SED1**	**SED2**	**SED3**	**ERL**	**ERM**
Cd	0.02	0.04	0.07	0.08	0.14	0.06	0.11	0.10	1.2	9.6
Cr	0.06	0.09	0.03	0.04	0.04	0.03	0.04	0.04	81	370
Cu	0.08	0.18	0.23	0.30	0.39	0.19	0.33	0.40	34	270
Hg	0.18	0.14	1.40	1.26	0.66	1.08	1.14	0.70	0.15	0.71
Ni	0.30	0.55	0.23	0.31	0.42	0.23	0.36	0.43	20.9	51.6
Pb	0.11	0.14	0.21	0.22	0.27	0.17	0.24	0.22	46.7	218
Zn	0.26	0.52	0.24	0.30	0.38	0.21	0.33	0.34	150	410
∑Metals	0.144	0.237	0.346	0.358	0.331	0.284	0.365	0.321	-	-
Acenaphthene [(1)]	0.133	0.166	0.004	0.002	0.0	0.012	0.0	0.0	16	500
Anthracene [(1)]	0.028	0.011	0.028	0.039	0.022	0.163	0.005	0.007	85.3	1100
Fluorene [(1)]	0.030	0.054	0.020	0.011	0.004	0.181	0.002	0.006	19	540
Naphthalene [(1)]	0.068	0.080	0.0	0.0	0.003	0.0	0.0	0.0	160	2100
Phenanthrene [(1)]	0.078	0.173	0.028	0.039	0.022	0.112	0.022	0.023	240	1500
Benzo(a)anthracene [(2)]	0.120	0.019	0.050	0.0	0.011	0.056	0.0	0.0	261	1600
Benzo(a)pyrene [(2)]	0.111	0.022	0.008	0.004	0.0	0.024	0.003	0.0	430	1600
Chrysene [(2)]	0.094	0.026	0.019	0.021	0.005	0.033	0.005	0.0	384	2800
Dibenzo(a,h)anthracene [(2)]	0.112	0.0	0.0	0.031	0.0	0.027	0.0	0.0	63.4	260
Fluoranthene [(2)]	0.056	0.015	0.018	0.015	0.003	0.012	0.007	0.003	600	5100
Pyrene [(2)]	0.099	0.042	0.024	0.019	0.008	0.013	0.010	0.005	665	2600
$\sum_{LMW}$PAHs $^{\Sigma(1)}$	0.120	0.178	0.021	0.027	0.015	0.145	0.013	0.015	552	3160
$\sum_{HMW}$PAHs $^{\Sigma(2)}$ (carcinogenic)	0.126	0.034	0.031	0.021	0.007	0.033	0.008	0.003	1700	9600
∑PAHs ($\sum^{(1)}$ + $\sum^{(2)}$)	0.035	0.020	0.008	0.006	0.002	0.017	0.003	0.002	4022	44792

Table 4. Concentrations and total sum of polycyclic aromatic hydrocarbons (PAHs, µg/kg dw) determined in the bulk fraction (<2 mm) of untreated sediments and experimental sediments from days 3 and 28. LMW PAHs: low molecular weight PAHs; HMW PAHs: high molecular weight PAHs. Asterisks indicate significant differences between laboratory and experimental conditions (z score, $p<0.05$); different letters indicate significant differences between experimental groups (z score, $p<0.05$).

	Untreated sediments		**Experimental sediments**					
			Day3			**Day28**		
	SED1	**SED3**	**SED1**	**SED2**	**SED3**	**SED1**	**SED2**	**SED3**
Acenaphthene (1)	66.7*	83.2*	2	1	0	6 a	0 b	0 b
Acenaphthylene (1)	0	0	0	0	0	0	0	0
Anthracene (1)	30.8	12.4	11	19	4	179 a*	6 b	8 b
Fluorene (1)	16	29.1*	11	6	2	98 a*	1 b	3 b
Naphthalene (1)	142*	167.5*	0	0	6	0	0	1
Phenanthrene (1)	116.5	260.1*	42	59	33	168 a	33 b	34 b
Benzo(b)fluoranthene	345.5*	82.1*	28 a	32 a	0 b	85 a	14 b	0 b
Benzo(k)fluoranthene	108.2	15.1*	26 a	0 b	1 b	55 a	0 b	0 b
Benzo(g,h,i)perylene	120.1*	49.8*	21	27	6	29	8	0
Benzo(e)pyrene	ND	ND	0	0	0	0	0	0
Indeno (1,2,3-c,d)pyrene	104.6*	19.1*	17	28	3	25	13	0
Perylene	ND	ND	0	0	0	0	0	0
Benzo(a)anthracene (2)	192*	31	80	0	17	89 a	0 b	0 b
Benzo(a)pyrene (2)	178.2*	35*	12	7	0	39 a	4 b	0 b
Chrysene (2)	262.2*	73.4*	53 a	58 a	13 b	91 a	13 b	0 b
Dibenzo(a,h)anthracene (2)	29.1	0	0 a	8 b	0 a	7 a	0 b	0 b
Fluoranthene (2)	287.5*	77.9*	93 a	78 a	14 b	60	35	16
Pyrene (2)	256.3*	109.6*	63	50	22	35	25	12
$\sum_{LMW}$PAHs $\Sigma^{(1)}$	372	552.3*	66	85*	45	451 a	40 b	46 b
$\sum_{HMW}$PAHs $\Sigma^{(2)}$ (carcinogenic)	1205.3*	326.9*	301	201	66	321 a	77 b	28 b
∑PAHs ($\sum^{(1)} + \sum^{(2)}$)	1577.3	879.2*	367	286	111	772 a	117 b	74 b
∑PAHs (16)	2255.7	1045.3*	459	373	121	966 a	152 b	74 b
Phe/Ant	3.78	20.98*	3.82 a	3.11 a	8.25 b	0.94*	5.50	4.25
Flr/Pyr	1.12	0.71	1.48 a	1.56 a	0.64 b	1.71 a	1.40 b	1.33 b*
$\sum_{LMW}$PAHs/$\sum_{HMW}$PAHs (%)	30.9	169.0	21.93	42.29	68.18*	140.5*	51.9	164.3
Ind / B[ghi]P	0.87	0.38	0.81*	1.04	0.50	0.86	1.63	-
Ind / (Ind + B[ghi]P)	0.47	0.28	0.45	0.51	0.33	0.46	0.62	-

In experimental SED1, Hg was at a concentration higher than ERM (and SQG-Q > 1) and Cu (always) and Pb (at day 28) were at concentrations between ERL and ERM values (Table 2). In experimental SED3, the concentrations of Cd, Cu, Hg, Ni, Pb and Zn were always between ERL and ERM values (Table 2). As a result, all experimental sediments had SQG-Q values above 0.1 for Cu, Ni, Pb and Zn; whereas SED2 (day 28) and SED3 (days 3 and 28) had SQG-Q values above 0.1 for Cd (Table 3). Considering the total sum of metals, SQG-Q values were in the range of 0.144 and 0.365 in the three sediments (Table 3). In untreated SED3, Ni, Cu and Zn concentrations were between ERL and ERM values, whilst no metal was at higher levels than their corresponding ERM in any untreated sediment.

Source SED3 had more total PAHs than source SED1 (Table 4; Larreta et al., 2012); however, after 3 d exposure conditions total PAHs diminished in both sediments (all levels below ERL; SQG-Qs < 0.1, Table 3); most drastically in SED3, resulting in levels much lower than in SED1 (Table 4). As a result, the concentrations of total $_{HMW}$PAHs at day 3 and of individual PAHs (Ant, Pyr, Phe) and total $_{LMW}$PAHs at day 28 were higher in SED1 than in SED3. Thus, levels of Ant and Fl detected in SED1 at day 28 were higher than the ERL values and SQG-Qs for Ant, Fle, Phe and Σ_{LMW} PAHs were 0.1-0.2 (Table 3). Finally, in untreated SED3 the concentrations of individual $_{LMW}$PAHs (Ace, Flu, Nph and Phe) was between ERL and ERM values, and in untreated SED1 only Ace (Table 4). This resulted in SQG-Q values above 0.1 for the sum of $_{LMW}$PAHs for untreated SED1 and SED3 and for $_{HMW}$PAHs in SED1 (Table 3).

3.2. Biological responses and toxicopathic effects

In total, 72 individuals were used. Length (22.5±2.1 cm), W (115.5±29.6 g), condition index K (1.0±0.1) and HSI (0.8±0.2) did not change throughout the experiment. Likewise, male GSI (GSI_{7d}=0.02±0.02; GSI_{28d}=0.04±0.02) and female GSI (GSI_{7d}=0.90±0.12; GSI_{28d}=0.96±0.16) did not differ between exposure groups although they tended to raise with time.

Table 5. Metals concentrations (µg/g dw) determined in liver samples collected from each experimental group at day 28. Different letters indicate significant differences between experimental groups (z score, $p<0.05$).

	Day28		
	SED1	**SED2**	**SED3**
Cd	2.29	2.1	1.46
Cr	0.66[a]	0.14[b]	0.11[b]
Cu	692	781	441
Fe	65.6	38.8	30.3
Hg	0.05	0.04	0.03
Mn	9.28	7.71	5.17
Ni	0.61	0.31	0.21
Pb	0.24[a]	0.09[b]	0.08[b]
Zn	62	54	40

The concentration of metals in liver after 28 d exposure tended to be higher in SED1 soles than in those exposed to SED2 and SED3, except for Cu (Table 5). Yet, significant differences were found only in the cases of Cr and Pb, which were at much higher concentrations in SED1 soles than in the other ones (e.g.: $[Cr]_{SED1}=([Cr]_{SED3}\times 6)$; $[Pb]_{SED1}=([Pb]_{SED3}\times 3)$).

Table 6. Summary of the 2-way ANOVAs performed to analyse the effects of sediments (d.f.: 2), time of exposure (d.f.: 1) and their combination ("Sediment × Time", d.f.: 2) on biomarkers and histopathology (lesion stages and indices) in *S. senegalensis* exposed to different sediments for 7 and 28 days. Logarithmic transformation was applied to CAT, GST, LP, Vv_L, S/V_L, Nv_L and Vv_{NL} (non-parametric variable). d.f.: degrees of freedom; F: Fisher's F; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

Parameter	Residual d.f.	F(Sediment)	F(Time)	F(S×T)
CAT	30	5.023*	19.922***	6.611**
GST	30	2.366	36.832***	0.074
AChE	29	2.407	0.474	3.739*
LP	64	8.380***	7.344**	2.234
Vv_L	61	4.265*	2.024	5.449**
S/V_L	61	8.985***	5.904	9.107***
Nv_L	61	10.312***	7.968**	10.267***
Vv_{NL}	69	5.854**	7.765**	3.214*
Epithelial lifting	72	11.171***	6.696*	1.059
I_{tot}	72	3.413*	4.078*	0.816
I_{liver}	72	4.185*	4.661*	0.432

No significant effect of S, T or S×T was detected for SOD, gills, liver and gonad lesion stages (with the exception of epithelial lifting), LS_{liver}, Igills, male Igon and female Igon.

Hepatic CAT activity was significantly affected by the sediment type (S), time (T) and sediment × time (S×T) interaction (2-way ANOVA; Table 6), varying from 10.75±3.78 µmol/min/mg prot in SED3 soles at day 7 to 31.61±11.30 µmol/min/mg prot in SED1 soles at day 28 (Figure 2A). Thus, at day 7, CAT activity was lower in SED3 soles than in other exposure groups and was lower than levels detected at day 28 whilst activities from other exposure groups remained constant. In contrast, hepatic GST activity only varied with T (2-way ANOVA; Table 6), being higher at day 28 than day 7 for all exposure groups and tended to be higher in SED1 soles (Figure 2B). Sediment type, T and S×T interaction did not exert any effect on hepatic SOD activity. Conversely, the S×T interaction affected brain AChE activity (2-way ANOVA; Table 6), which raised in SED3 after 28 d treatment (Figure 2C).

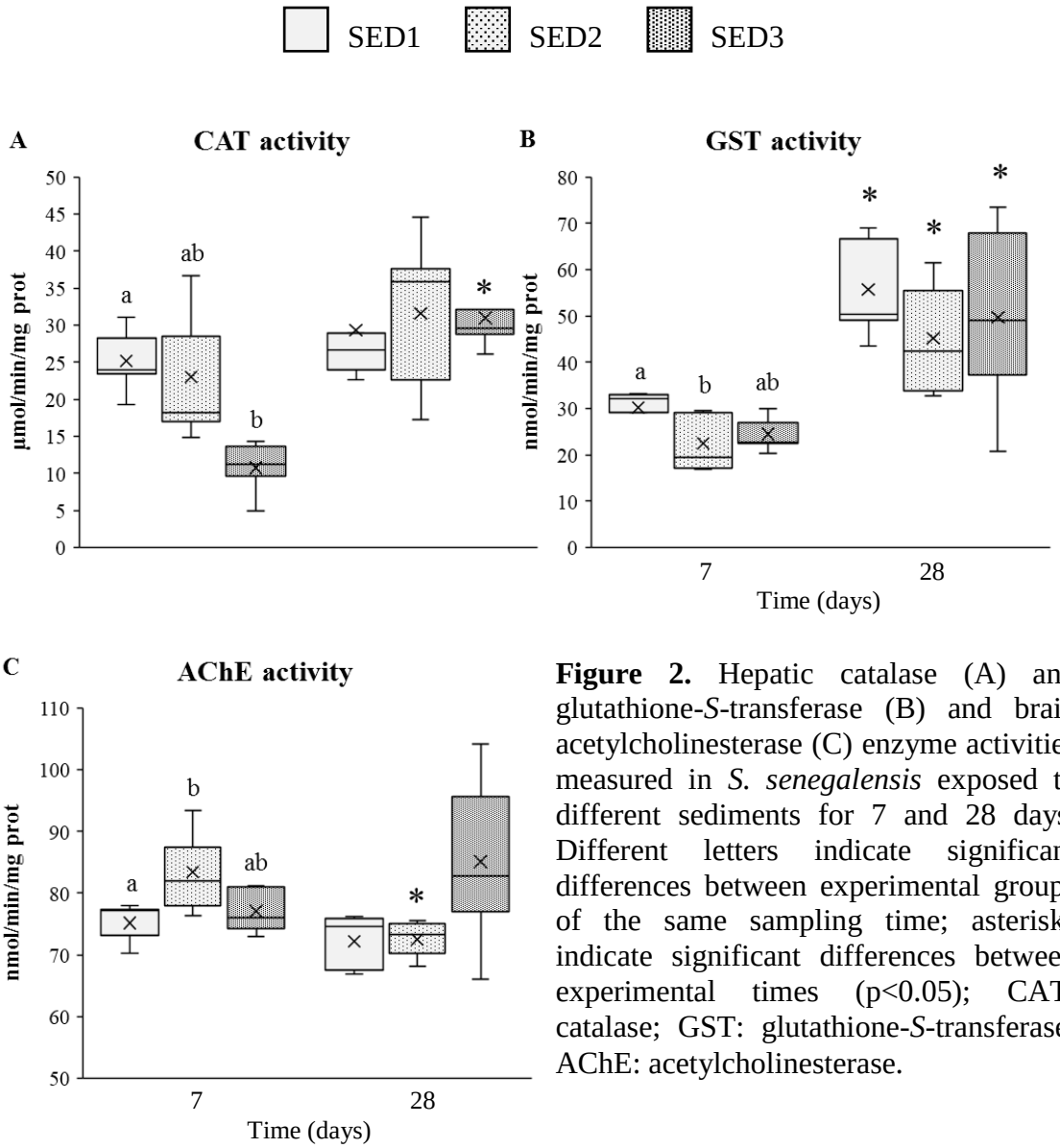


Figure 2. Hepatic catalase (A) and glutathione-*S*-transferase (B) and brain acetylcholinesterase (C) enzyme activities measured in *S. senegalensis* exposed to different sediments for 7 and 28 days. Different letters indicate significant differences between experimental groups of the same sampling time; asterisks indicate significant differences between experimental times ($p<0.05$); CAT: catalase; GST: glutathione-*S*-transferase; AChE: acetylcholinesterase.

Lysosomal LP was significantly affected by S and T (2-way ANOVA; Table 6); ranging from 17.98±4.76 min in SED1 soles at day 7 to 5.64±0.18 min in SED3 at day 28 (Figure 3A). At day 7, LP was lower in soles exposed to SED3 than in those exposed to SED1 and SED2 and remained low at day 28, when the LP values dropped, as well in soles exposed to SED1 and SED2 (Figure 3A). Lysosomal Vv_L and S/V_L were significantly affected by S and S×T; whilst Nv_L was affected by S, T and S×T (2-way ANOVA; Table 6). Thus, S/V_L and Nv_L were lower in SED2 and SED3 than SED1 at day 7 and in SED3 than in SED1 at day 28 (Figure 3B). At day 28, in soles exposed to SED3, Vv_L raised and S/V_L and Nv_L decreased (Figure 3B). Vv_{NL} was significantly affected by S, T and the S×T interaction (2-way ANOVA; Table 6); ranging from 0.032±0.021 $\mu m^3/\mu m^3$ (SED1, at day 7) to 0.195±0.029 $\mu m^3/\mu m^3$ (SED3, at day 28; Figure 3E). Vv_{NL} did not differ between experimental groups at day 7 but increased in soles exposed to SED3 at day 28.

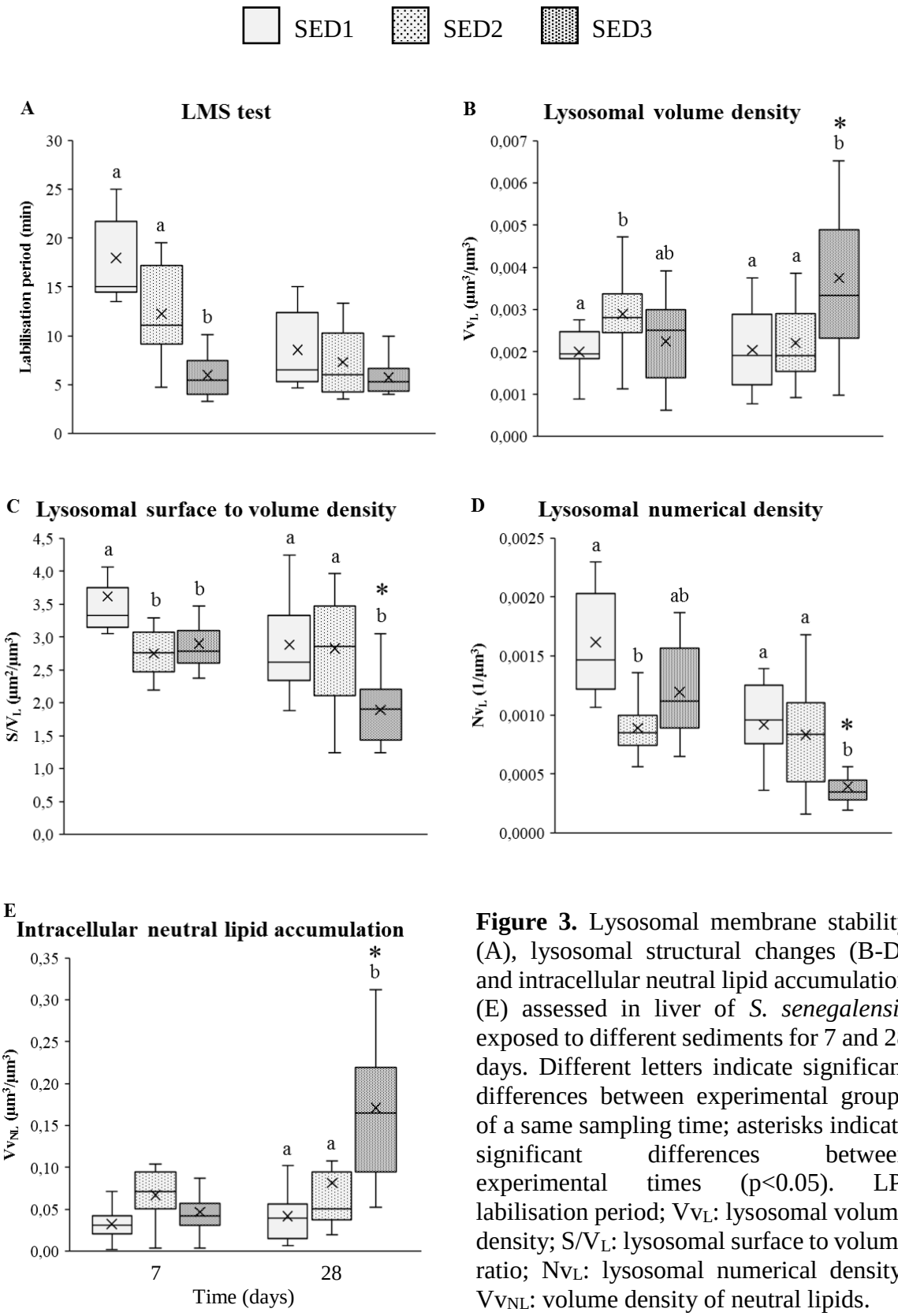


Figure 3. Lysosomal membrane stability (A), lysosomal structural changes (B-D) and intracellular neutral lipid accumulation (E) assessed in liver of *S. senegalensis* exposed to different sediments for 7 and 28 days. Different letters indicate significant differences between experimental groups of a same sampling time; asterisks indicate significant differences between experimental times ($p<0.05$). LP: labilisation period; Vv_L: lysosomal volume density; S/V_L: lysosomal surface to volume ratio; Nv_L: lysosomal numerical density; Vv_{NL}: volume density of neutral lipids.

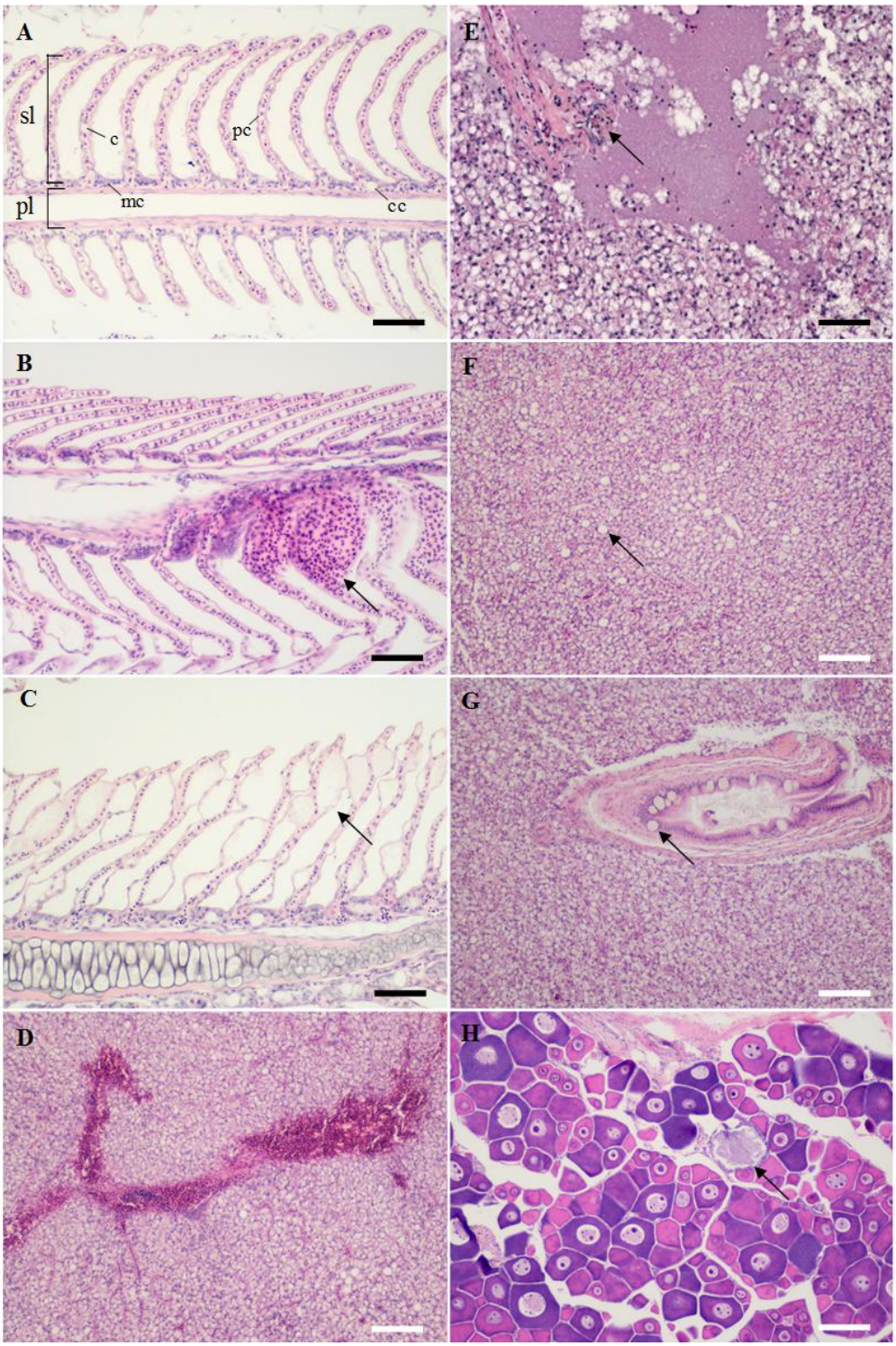


Figure 4. Histological sections (5 µm) of *S. senegalensis* exposed to different sediments for 7 and 28 days, stained with hematoxylin-eosin. (A) Normal gills showing well-arranged secondary lamellae. (B) Capillary aneurysm (arrow) in gills. (C) Severe epithelial lifting (arrow) in gills. (D) Hyperaemia in liver (note masses of erythrocytes in blood vessels). (E) Severe necrosis and presence of a MMCs (arrow) in liver. (F) Fat vacuolation in hepatocytes (arrow). (G) Hydropic vacuolation of epithelial cells of bile duct (arrow). (H) Immature female gonad showing a case of atresia (arrow). c: capillary; cc: chloride cell; mc: mucous cell; pc: pillar cell; pl: primary lamella; sl: secondary lamellae. Black scale bar: 50 µm; white scale bar: 100 µm.

Table 7. Lesions prevalence (%) and means and standard errors of lesions stages (n=12; lesion intensity when detected) for gill histopathological lesions identified in *S. senegalensis* collected from each experimental group at days 7 and 28. Different letters indicate significant differences between experimental groups ($p<0.05$).

		Day7			Day28		
Gill lesions	***w***	**SED1**	**SED2**	**SED3**	**SED1**	**SED2**	**SED3**
Circulatory disturbances							
Lamellar capillary aneurysm	1	100.0[a] (2.2±0.2)	91.7[b] (2.0±0.0)	100.0[a] (2.2±0.2)	100.0 (2.0±0.0)	100.0 (2.0±0.0)	100.0 (2.0±0.0)
Regressive changes							
Epithelial lifting	1	100.0 (3.0±0.4[a])	100.0 (4.3±0.5[b])	100.0 (5.0±0.4[b])	100.0 (4.0±0.3[a])	100.0 (5.5±0.3[b])	100.0 (5.2±0.3[b])
Progressive changes							
Lamellar fusion	1	66.7 (2.5±0.3)	58.3 (2.3±0.3)	75.0 (2.0±0.0)	58.3 (2.0±0.0)	83.3 (2.0±0.0)	75.0 (2.0±0.0)
Epithelial hyperplasia	2	100.0 (3.8±0.5)	100.0 (3.8±0.5)	100.0 (3.2±0.5)	100.0 (3.5±0.4)	100.0 (3.5±0.4)	100.0 (3.7±0.6)

w: lesion importance factor.

In all the experimental groups the histological structure of the gills was readily recognised, exhibiting well-arranged secondary lamellae along the gill filaments and an epithelium composed of pavement, mucous and chloride cells (Figure 4A). Yet, practically all the examined soles presented lamellar capillary aneurysm at mild lesion stage (Figure 4B; Table 7), epithelial hyperplasia at moderate lesion stage and epithelial lifting at high lesion stage (Figure 4C; Table 7). In the case of epithelial lifting, the lesion stage was significantly affected by S and T but not by their interaction S×T (2-way ANOVA; Table 6) with the lowest value found in SED1 soles. No tumours were detected in gills.

Overall the liver presented a normal histological appearance with a conspicuous bi-layer of hepatocytes aligned with sinusoids, and with sparse bile ducts and blood vessels. The exocrine pancreatic tissue was occasionally observed in the parenchyma. Hyperaemia was the only circulatory disturbance identified (Figure 4D) showing the highest lesion stage and prevalence in SED3 soles (Table 8). Similarly, lymphocytic infiltration was only identified in soles exposed to SED3 for 28 d (8.3%; 0.17±0.17). In contrast, the lesion stage of MMCs did not vary during the exposure period, yet the lesion prevalence increased with time in all experimental groups (Table 8). The highest lesion stage and prevalence of necrosis (Figure 4E) were found in soles exposed to SED3. Fat vacuolation of hepatocytes was identified in all animals (Figure 4F) with a higher lesion score detected in soles exposed to SED3 (Table 8). Hydropic vacuolation of epithelial cells of bile ducts and concentric periductal fibrosis were seldom detected (Figure 4G). The highest lesion stage of concentric periductal fibrosis was recorded after 28 d, particularly in SED3 soles. No neoplastic lesions were found in liver.

Upon microscopic examination of gonad tissue most soles were at an early stage of gamete development. Males presented immature testis (24.2%) or early spermatogenesis

Table 8. Lesions prevalence (%) and means and standard errors of lesions stages (n=12; lesion intensity when detected) for liver histopathological lesions identified in *S. senegalensis* collected from each experimental group at days 7 and 28. Different letters indicate significant differences between experimental groups ($p<0.05$); asterisks indicate significant differences between sampling days (Pearson's Chi test, $p<0.05$).

		Day7			Day28		
Hepatic lesions	***w***	**SED1**	**SED2**	**SED3**	**SED1**	**SED2**	**SED3**
Circulatory disturbances							
Hyperaemia	1	8.3[a] (2.0±0.0)	8.3[a] (2.0±0.0)	16.7[b] (2.0±0.0)	8.3 (2.0±0.0)	16.7 (2.0±0.0)	33.3 (2.0±0.0)
Inflammatory responses							
MMCs	1	16.7[a] (2.0±0.0)	8.3[b] (2.0±0.0)	8.3[b] (2.0±0.0)	33.3 (2.0±0.0)	50.0* (2.0±0.0)	25.0 (2.0±0.0)
Regressive changes							
Necrosis	3	50.0 (2.3±0.3)	16.7 (2.0±0.0)	83.3 (2.0±0.0)	50.0 (2.3±0.3)	33.3 (2.5±0.5)	83.3 (2.4±0.3)
Progressive changes							
FV of hepatocytes	1	100.0 (2.8±0.4)	100.0 (2.8±0.5)	100.0 (3.7±0.4)	100.0 (3.2±0.3)	100.0 (3.7±0.3)	100.0 (4.0±0.3)
HV of epithelial cells of bile ducts	2	8.3[a] (2.0±0.0)	8.3[a] (4.0±0.0)	0.0[b] (0.0)	8.3 (2.0±0.0)	8.3 (2.0±0.0)	8.3 (4.0±0.0)
CPF of bile ducts	2	16.7[a] (2.0±0.0)	0.0[b] (0.0)	0.0[b] (0.0)	33.3[a] (2.0±0.0)	33.3[a]* (2.0±0.0)	25.0[b] (2.7±0.7)

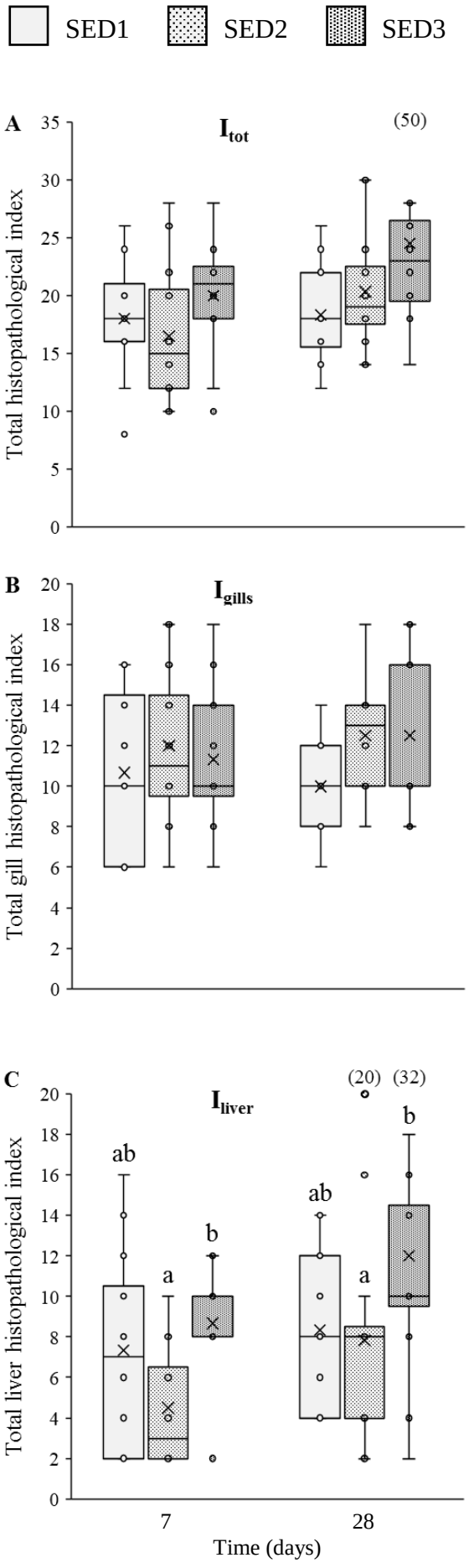
w: lesion importance factor; MMCs: Melanomacrophage Centres; FV: Fat Vacuolation; HV: Hydropic Vacuolation; CPF: Concentric Periductal Fibrosis.

stage (75.8%); and although one case of early vitellogenesis (Stage II) was identified, most females (91.1%) presented primary growth oocytes (Stage I). Testis lesions were scarce and only few cases of necrosis (20.0%) and lymphocytic infiltration (33.3%) were recorded in SED1. Atresia was the most frequent alteration in ovary, being present in all experimental groups (>25.0%; Table 9). The highest lesion stage and prevalence for atresia were detected in soles exposed to SED3 (Table 9). Granulomatosis and oocyte necrosis and lipidosis were rarely found (3.1%). No tumours were detected in gonad tissue.

Table 9. Lesions prevalence (%) and means and standard errors of lesions stages for female gonad histopathological lesions identified in *S. senegalensis* collected from each experimental group at days 7 and 28. Different letters indicate significant differences between experimental groups ($p<0.05$).

		Day7			Day28		
		SED1	**SED2**	**SED3**	**SED1**	**SED2**	**SED3**
Gonad lesions in females	***w***	n=5	n=3	n=6	n=8	n=4	n=6
Inflammatory responses							
Lymphocytic infiltration	2	20.0[a] (2.0±0.0)	0.0[b] (0.0)	16.7[a] (2.0±0.0)	12.5 (2.0±0.0)	25.0 (2.0±0.0)	16.7 (2.0±0.0)
Regressive changes							
Atresia	3	40.0[a] (2.0±0.0)	33.3[a] (2.0±0.0)	83.3[b] (2.0±0.0)	37.5 (2.0±0.0)	25.0 (2.0±0.0)	66.7 (2.0±0.0)

w: lesion importance factor.



LS_{liver} did not differ between exposure groups. The lowest LS_{liver} was measured in SED1 after 7 d experimentation (1.58±0.26) and the highest in SED3 after 28 d (2.33±0.26). S and T exerted significant effects on I_{tot} and on I_{liver} but not on I_{gill} (2-way ANOVA; Table 6). Thus, I_{tot} and I_{liver} were higher in SED3 than in SED1 and SED2 (Figure 5A and C), whilst I_{gill} did not vary between experimental groups (Figure 5B).

The IBR/n index ranged from 0.16 in soles exposed to SED1 for 7 d to 3.05 in soles exposed to SED3 for 28 d (Figure 6). Overall, IBR/n index was higher at day 28 than at day 7 and higher in SED3 than in SED 1 and SED 2 at day 28 (Figure 6).

Figure 5. Total index (I_{tot}), gill index (I_{gills}) and liver index (I_{liver}) of *S. senegalensis* exposed to different sediments for 7 and 28 days. Different letters indicate significant differences between experimental groups of the same sampling time; asterisks indicate significant differences between experimental times ($p<0.05$).

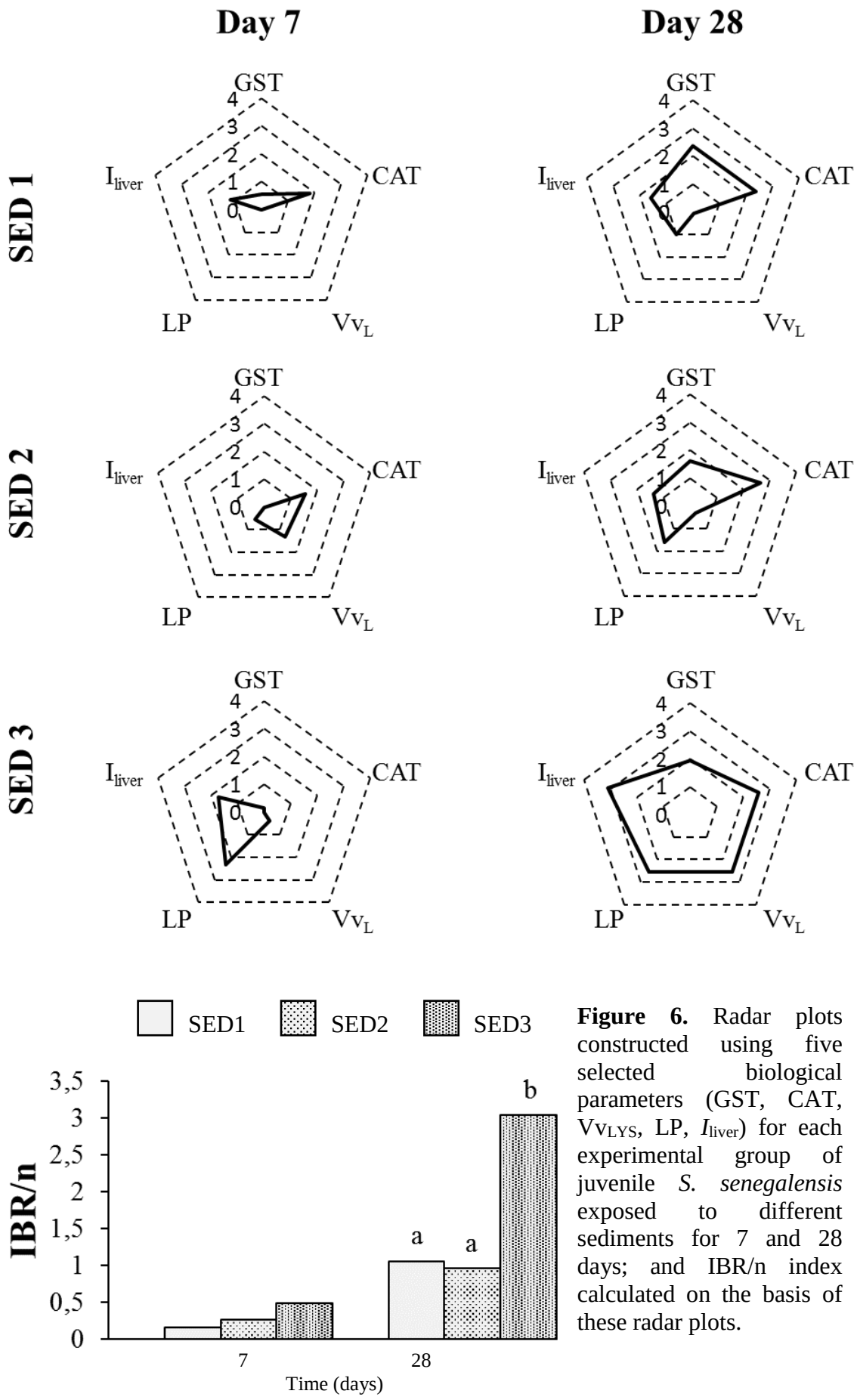


Figure 6. Radar plots constructed using five selected biological parameters (GST, CAT, Vv_{LYS}, LP, I_{liver}) for each experimental group of juvenile *S. senegalensis* exposed to different sediments for 7 and 28 days; and IBR/n index calculated on the basis of these radar plots.

4. Discussion

Consequences of the experimental setup on sediment properties

The sediments used in this study presented different profiles and levels of chemical contaminants and were characterised by different degrees of potential toxicity. For instance, Cd and Hg were present in the source sediments in Plentzia and Pasaia but the overall levels of metals were higher in Pasaia than in Plentzia (Solaun et al., 2009). Pasaia harbour is largely contaminated with Cd and other heavy metals resulting from the geological composition of the basin and mining and industrial activities (Belzunce et al., 2004). Plentzia estuary is one of the least contaminated along the coast of Bizkaia (Belzunce et al., 2004); however, upstream mining and industrial activities may be a source for metals in sediments (Borja et al., 2002; Cortada and Collins, 2013). Likewise, sediments in Pasaia have more total PAHs than in Plentzia (Solaun et al., 2009; Larreta et al., 2012). Indeed, PAH levels in Pasaia are amongst the highest recorded in Basque estuaries and seem to be related with paper mill and fishing fleet activities (Legorburu and Cantón, 1991; Franco et al., 2001). According to different diagnostic ratios Phe/Ant and Flr/Pyr (Baumard et al., 1998; Soclo et al., 2000; Neff et al., 2005), Pasaia sediment PAHs would be of pyrolytic origin whereas Plentzia sediment PAHs would be of petrogenic origin. In agreement, metal levels in untreated sediments used in this study were higher in SED3 (from Pasaia) than in SED1 (from Plentzia), and showed a distinct profile depending on the source (Cd, Cu, Pb, and Zn in SED3; and Cr, Cu, Fe, Hg, Mn, Ni, Pb and Zn in SED1). Nevertheless, metals in untreated sediments only posed a minor toxic risk since Ni, Cu and Zn were between ERL and ERM values in SED3 and no metal was at levels beyond ERM values in any case. Likewise, the concentration of individual PAHs in untreated SED3 was between ERL and ERM values, like Ace in the case of untreated SED1. Based on the sediment quality guidelines provided by Long et al. (1995), the presence of pollutants in the different sediments at levels exceeding the ERL and ERM levels suggests potential biological effects.

Metal contaminant profiles in experimental sediments were comparable to those aforementioned for untreated sediments. Overall, concentrations were moderately low since e.g. SQG-$Q_{\Sigma Metals}$ values were around 0.3 (Long et al., 1995). Yet, Hg, which was found to be at moderately toxic levels in untreated SED1 and SED2, was at concentrations that would result toxic (SQG-Q>1) in those experimental sediments. Likewise, in comparison with untreated sediments, the levels of total PAHs, which were low (SQG-Qs<0.1), resulted in even lower levels in experimental sediments, more severely in SED3. As a result, the concentrations of total and certain individual PAHs were higher in SED1 than in SED3, unlike for the case of source sediments in which PAH levels were higher in Pasaia than in Plentzia (Legorburu and Cantón, 1991; Franco et al., 2001). This apparent inconsistency may be explained by changes in chemical profile of sediments during transportation, storage and manipulation as a result of the changes in physicochemical properties (redox potential, OM and granulometry) and biological activity (bioturbation and biotransformation) (Eggleton and Thomas, 2004). Thus, changes in granulometry recorded in SED1 and SED3 suggested a loss of sediment during

the experiment, potentially related to the experimental continuous water flow and the presence of soles. This may imply a subsequent loss of chemicals from the sediments, in agreement with chemical data showing lower PAHs concentrations in experimental sediments compared to untreated and source sediments. Granulometry also indicated a quicker sediment loss in SED3 than SED1, suggesting a greater decrease in pollutant concentrations. In the case of organic contaminants, PAHs can also be degraded by induction of the activity of PAH degrading aerobic microorganisms as suggested by the shift from negative to positive values in redox potential recorded in SED3 (Eggleton and Thomas, 2004; DeLaune and Reddy, 2005). Besides, an increase in redox potential might be related with an enhanced mobilisation of sediment metals that are released to the water column (Zhang et al., 2014). Consequently, if contaminants were importantly released from the SED3, waterborne exposure cannot be disregarded for soles under the present experimental conditions, even though contaminant levels in SED3 are low.

Biological responses and toxicopathic effects

Biometry, condition, sex and reproductive status did not differ between exposure groups. Individuals were shown to be juveniles with gonad at an early stage of gamete development.

In agreement with previous field studies (Vicente-Martorell et al., 2009; Oliva et al., 2012b), metals such as Cr, Fe, Mn, Ni and Pb were accumulated in the liver under the present exposure conditions. Levels of sediment contaminants reported in these field studies were considerably higher than metal concentrations measured in the present experiment. However, the concentrations and the final accumulation ranking of metals (Cu>Fe>Zn>As>Cd>Pb) were similar (Oliva et al., 2012b). Although the levels of metals, other than Hg, are comparable in the experimental sediments, the concentrations of Cr and Pb in liver were higher in SED1 exposed soles than in those exposed to SED2 and SED3. Moreover, although Hg levels in SED1 and SED2 were higher than in SED3, Hg does not seem to be differentially accumulated in liver.

Oxidative stress

Induction of antioxidant enzymes has been reported in a variety of fish species, including sole, as a signal of antioxidant defence activation in response to exposure to diverse contaminants (Regoli and Principato, 1995;Pedrajas et al., 1996; Pinto et al., 2003; van der Oost et al., 2003; Atli et al., 2006; do Carmo Langiano and Martinez, 2008; Fonseca et al., 2011a; dos Santos Carvalho et al., 2012; Ghribi et al., 2019).

CAT activity in fish liver may respond in a dose dependent manner (either induction or inhibition) albeit the type of response may be different depending on the pollutant and on the duration of the exposure (Pedrajas et al., 1996; Regoli et al., 2002). Accordingly, in the present study hepatic CAT activity varied with S, T and S×T with values ranging approximately from 10 to 30 µmol/min/mg prot. Thus, with the exception of soles exposed to SED3 for 7 days, the values of CAT activity presently recorded resembled those measured in control sole juveniles maintained under laboratory conditions (Solé et al., 2008; Oliva et al., 2012b; Chapters 3 and 4). However, in individuals exposed to

SED3 for 7 days, CAT activity (10.75±3.78 µmol.min^{-1}.mg^{-1}prot) was reduced to levels comparable to those recorded on extreme exposure to waterborne Cd (Chapter 3) and BaP (Chapter 4) or to those recorded in wild sole juveniles from highly contaminated sites (Oliva et al., 2012b). Conversely, CAT activity in fish liver is known to be induced as a part of the antioxidant defence mechanisms elicited on exposure to pollutants (Di Giulio et al., 1989). Thus, the reduction observed herein may suggest that SED3 pollutants exceeded the antioxidant capacity of soles leading to toxic damage and enzyme activity inhibition, as previously reported (Roméo et al., 2000; Kalman et al., 2010). It seems that during the experimental setup higher levels of waterborne metals and PAHs are mobilised from SED3 than from SED1, as above discussed; which might explain the more marked effect of SED3 on CAT in comparison with SED1 on day 7. Interestingly, CAT levels in SED3 exposed soles return to levels comparable to those recorded in the other exposure groups. Since the release of pollutants from sediment to water seems to be lowered beyond 7 experimental days, it is conceivable that soles are less exposed to waterborne pollutants during this experimental period. Alternatively, some acclimatization cannot be disregarded, as the antioxidant enzyme responses can be either attenuated or reversed when exposure to pollutants is long-lasting (Regoli and Principato, 1995).

Hepatic GST is enhanced in juvenile soles and other fishes exposed to a variety of pollutants both through waterborne and sediment exposure (Regoli et al., 2002; Salamanca et al., 2008; Ghribi et al., 2019; Chapters 3 and 4). Presently, however, unlike in the case of CAT, GST was equally enhanced on exposure to the three experimental sediments raising from values around 20-35 µmol.min^{-1}.mg^{-1}prot at day 7 to up to 70 nmol.min^{-1}.mg^{-1}prot at day 28.

Likewise, SOD activity may be enhanced in fish liver exposed to pollutants (Pedrajas et al., 1996; Regoli et al., 2002); however, hepatic SOD activity did not respond to the present experimental treatments, average activity being ≈115 nmol.min^{-1}.mg^{-1}prot. Overall, SOD responses to environmental stressors in fish liver have been controversial and previous studies have reported both enzyme induction and inhibition (Wu et al., 2006; dos Santos Carvalho et al., 2012; Souid et al., 2013).

Neurotoxicity

AChE activity is known to be inhibited by chemicals; especially but not only by pesticides (van der Oost et al., 2003; Davies and Vethaak, 2012). Presently, brain AChE activity did not show remarkable differences between experimental groups, activity values being similar to those recorded in control juvenile soles in other experimental setups (≈ 80 nmol.min^{-1}.mg^{-1}prot; Chapters 3 and 4). Thus, neurotoxic chemicals might be absent or only present at non-effective concentrations in the experimental sediments; or maybe the exposure time was too short to elicit any change in this enzyme activity.

Lysosomal responses

Fish liver lysosomes are key organelles for sequestration and detoxification of chemical pollutants resulting in changes in lysosomal structure and membrane integrity used as core biomarkers in biological effects assessment (Köhler et al., 2002; Alvarado et al.,

2005; Baršienė et al., 2006; Zorita et al., 2008; ICES, 2011; Burgeot et al., 2017; Moore et al., 2013; Le Croizier et al., 2019).

Presently, lysosomal membrane stability in liver of sole juveniles exposed to SED3 was reduced after 7 days whereas a similar reduction was observed in SED1 and SED2 exposed soles only at day 28. In sole exposed to SED1 for 7 days, LP was comparable to that recorded in healthy fish in previous studies (Köhler et al., 1992; Köhler and Pluta, 1995; Broeg et al., 1999, 2002; Viarengo et al., 2007; Zorita et al., 2008). Likewise, the lowest LP values found herein resembled those reported in sole and other fish species exposed to pollutants (Baršienė et al., 2006; Zorita et al., 2008; Burgeot et al., 2017; Chapters 3 and 4). According to Köhler et al. (1992), this destabilisation of the lysosomal membrane recorded would reflect a non-specific toxic effect distinctly exerted by the different sediments; SED3 being the most toxic. Interestingly, this degree of effect (LP<10 min) has been suggested to be linked to further potential pathological alterations at tissue level (Köhler, 1990; Köhler et al., 2002; Viarengo et al., 2007).

Early lysosomal responses to pollutants in aquatic animals include increase in size and reduction in numbers, which is interpreted as lysosomal enlargement (Köhler et al., 1992; Cajaraville et al., 1995; Marigómez et al., 2005; Alvarado et al., 2005; Dagnino et al., 2007; Izagirre and Marigómez, 2009). Presently, Vv_L (≈0.002-0.004 $\mu m^3/\mu m^3$) was higher and S/V_L (≈2-4 $\mu m^2/\mu m^3$) and Nv_L (≈0.0005-0.0020 $1/\mu m^3$) lower in all the treatments than those reported for control sole juveniles in other studies (Vv_L: ≈0.0003 $\mu m^3/\mu m^3$; S/V_L: ≈5 $\mu m^2/\mu m^3$; Nv_L: ≈0.0004 $1/\mu m^3$; Chapters 3 and 4). Thus, a certain lysosomal enlargement (low S/V_L and Nv_L values) can be envisaged in all the experimental treatments. Moreover, additional lysosomal enlargement was observed in SED2 and SED3 at day 7 and more markedly in SED3 at day 28, when Vv_L values resulted to be extremely high. In agreement with lysosomal membrane destabilisation, lysosomal enlargement would also indicate that all the experimental sediments exert a non-specific toxic effect, which is extreme in soles exposed to SED3 for 28 days.

Similarly, intracellular accumulation of neutral lipids was only observed in soles exposed to SED3 for 28 days, whilst levels recorded in the other groups were similar to values recorded in control soles in other experimental studies (Chapter 3). Lipid accumulation in fish liver has been recorded previously in response to exposure to contaminants and was related to the storage of liposoluble xenobiotics (PAHs and organochlorides) in the organ (Köhler, 1990).

Histopathology

The toxicological relevance of histopathological alterations can be determined upon the use of categorized lesions as indicators for monitoring biological effects of contaminants in fish (Feist et al., 2004; Lang et al., 2006). Many of these alterations were identified in sole juveniles in the present study. Overall, the lesion stage and prevalence of histopathological alterations and the related histopathological indices recorded in gills, liver and gonad advocate for the suitability of juvenile *S. senegalensis* histopathology as a tool for assessing the biological effects of contaminated sediments.

Lamellar capillary aneurysm, epithelial hyperplasia and lifting were found in practically all the gills of all the specimens examined, with prevalence values close to 100%. However, lesion stages were mild for lamellar capillary aneurysm, moderate for epithelial hyperplasia and high for epithelial lifting, especially in SED2 and SED3, and more remarkably at day 28. These lesions could be related with the above discussed different toxicity of the sediments. Indeed, the occurrence of these lesions in gills has been reported to be associated to the presence of pollutants in the sediment (Arellano et al., 1999; Stentiford et al., 2003; Jímenez-Tenorio et al., 2008; López-Galindo et al., 2010a, 2010b; Oliva et al., 2013; Chapter 1). Likewise, hyperplasia in fish gills has been correlated with the waterborne levels of PAHs and pesticides (Noreña-Barroso et al., 2004). Gills are usually the first contact and the primary and main uptake site for contaminants (Mallat, 1985) and therefore histopathological lesions in gills are considered indicators of recent stress situation (Costa et al., 2009a; Kalman et al., 2010). On the other hand, exposure to suspended sediment and associated turbidity are known to alter the gill structure leading to further effects on the physiology and condition of the fish (Lowe et al., 2015; Cumming and Herbert, 2016; Hess et al., 2017). Therefore, although it cannot be disregarded that pollutants in the experimental sediments can cause these lesions, it is not unlikely that they might also be caused by sediment itself, especially if considering that sole juveniles (acquired in a farm) had not been kept on sediment before the present experiment.

In liver, no signs of neoplasia were found and hydropic vacuolation of epithelial cells of bile ducts and concentric periductal fibrosis were only eventually recorded. However, mild lesion stages of hyperaemia, lymphocytic infiltration, MMCs and necrosis were found. These lesions have been previously described in sole, both after laboratory exposure to pollutants and in field studies (Costa et al., 2011; Oliva et al., 2013; Zorita and Cuevas, 2014; Chapters 1, 3 and 4). Mild occurring lesions can be found in normal liver and may be less important in pollution effects assessment (van Dyk et al., 2012; Feist et al., 2004); however it has been recommended that they should be always recorded (Feist et al., 2004). The prevalence of MMCs did not vary between experimental sediments but was higher at day 28 than at day 7 in all the cases. The highest prevalence of hyperaemia, lymphocytic infiltration and necrosis was recorded in soles exposed to SED3 for 28 days. The prevalence of hyperaemia and necrotic foci has been described to raise moderately in gills and liver on exposure to a variety of pollutants including e.g. Cd, pesticides and B(a)P (Au, 2004; Noreña-Barroso et al., 2004; Oliveira Ribeiro et al., 2005; van Dyk et al., 2007; Chapters 3 and 4). Thus, the higher prevalence of these lesions may be related to a higher toxicity elicited by the pollutants present in or released from SED3 than in SED1 and SED2.

In gonad, no tumour was recorded whilst lymphocytic infiltration and necrosis in testis and granulomatosis, necrosis and lipidosis in oocytes were only rarely found. In contrast, oocyte atresia was most frequently found in soles exposed to SED3. These lesions were also reported in soles and other fish species subject to environmental stress (Blazer, 2002; Reynolds et al., 2003; Cuevas et al., 2015a; Chapters 1, 3 and 4). Oocyte atresia may occur during the normal reproductive cycle but enhanced atresia has been reported in

response to environmental stressors (Witthames et al., 1995; Chapter 1). Therefore, the presently recorded higher prevalence of oocyte atresia found in SED3 exposed soles might be attributed, like mentioned herein for other biological effects endpoints, to a higher toxicity of sediment in comparison with SED1.

Histopathological indices may contribute to establish cause-effect relationships between pathology in sole and pollutants in sediments by integrating the biological significance (w: lesion importance factor) and the extension of the lesions (Costa et al., 2009a). Presently, the LS_{liver} index developed for flounder (Lang et al., 2006) was applied for hepatic lesions and the weighted histopathological indices (Bernet et al., 1999) adapted for sole by Costa et al. (2009a) were used for gills and liver. The LS_{liver} did not reveal any relevant difference between experimental groups; conversely, some differences were found in weighted histopathological indices. Thus, I_{liver} and I_{tot} were higher in SED3 than in SED1 and SED2 and I_{gills} was seemingly higher in all the experimental groups (I_{gills}=6-18) than in control soles of other experimental setups (I_{gills}<8; Chapters 3 and 4). As a result and in agreement with other endpoints studied herein, I_{gills} seems to indicate that exposure to the three sediments exert a certain biological effect on sole juveniles under the present experimental conditions. Moreover, I_{liver} was higher on exposure to SED3 for 28 days (I_{liver} =12.00±2.23) than in other experimental groups of the present study (I_{liver}=4.5-8.5), in control groups in other experimental setups (average I_{liver}<11; Chapters 3 and 4) and in reference or less polluted sites in field studies (average I_{liver}<10; Costa et al., 2009a; 2011). Therefore, I_{liver} values also support that SED3, and especially at day 28, is the most toxic of the experimental sediments. Indeed, comparable I_{liver} values were reported in fish exposed to pollutants in the lab and collected from polluted sites (Costa et al., 2009a; Cuevas et al., 2015b; Chapter 1). Finally, since in the present study I_{liver} was the main contributor to I_{tot}, both indices reflected essentially the same.

Integrative assessment

The integration of biological responses into an IBR/n index aims to illustrate the overall effect of each treatment using five key biological parameters: GST and CAT activities, Vv_{LYS}, LP and liver histopathology. The IBR/n values confirmed that exposure to the three sediments caused significant biological responses and toxicopathic effects of diverse magnitude (IBR/n_{SED3}>IBR/n_{SED2}>IBR/n_{SED1}). Thus, according to the IBR/n index SED3 was the experimental sediment that exerted the most marked effect on sole health status, which was evident already after 7 days treatment. In the cases of SED1 and SED2, however, the impact on sole health was seemingly delayed and observed only at day 28. Moreover, the differences in the shapes of the radar plots reflected the presence of dissimilar profiles and levels of pollutants in the different exposure groups. These, as above discussed, can be related to differences between the source sediments but also to the changes observed along the present experimental setup in the physicochemical properties and contaminant burdens of the sediments. Thus, whereas in SED1 the main contributors to IBR/n at day 28 were biochemical responses related to antioxidant defence (GST and CAT), all the levels of biological response and especially toxicopathic effects and their early signal, the lysosomal responses, contributed to IBR/n in SED3, with SED2

in between (GST, CAT and LP). The IBR approach combining biomarkers and histopathology has been previously applied for a general scoring of the health status in fish exposed to a variety of waterborne pollutants (Oliveira et al., 2009; Kim et al., 2010; Serafim et al., 2012; Valerio-García et al., 2017; Iturburu et al., 2018), with results fully comparable to ours. The use of this index herein contributed to an overall evaluation of biological responses to contaminated sediments and minimized the potential uncertainty due to the inherent variability of individual biomarkers (Marigómez et al., 2013; Baudou et al., 2019).

Concluding remarks

Sediments used in this present study differed in pollutant concentrations and, although they were present at mild concentrations, the mixture of chemicals indicated different degrees of potential toxicity. Additionally, sediments reacted differently in experimental conditions suggesting distinct release of pollutants from each sediment, depending on their physicochemical properties. Chemical analysis demonstrated the complexity of sediments as source of pollutants, characterised by an alterable toxicity. Differences in toxicity associated with each sediment was supported by the biological responses elicited in sole juveniles. Thus, enhanced hepatic GST activity showed toxicity from all experimental sediments whilst CAT inhibition revealed a more pronounced toxicity in SED3. Similarly lysosomal responses (lysosomal enlargement, membrane destabilisation and changes in lysosomal content) indicated a non-specific toxic effect from all the sediments, more severe in the case of SED3 exposure. Accordingly, gill alterations were equally detected in all experimental groups whilst liver histopathology permitted to highlight a higher toxicity exerted by SED3.

In conclusion, the integration of biological responses assessed at different levels of biological complexity and based on diverse biological endpoints in sole allowed to highlight and differentiate toxicity profiles of contaminated sediments. Toxicopathic effects were related with the contamination reported for source sediments indicating higher toxicity from SED3 than SED1 and SED2, unlike suggested by chemical levels detected in experimental sediments after the exposure period. Thus, although levels of sediment contaminants changed under experimental conditions, the overall biological responses were representative of the source sediment toxicity; seemingly linked to the release of contaminants to the water column during experimentation. The present study confirms the suitability of whole-sediment toxicity assays to elucidate the association between the presence of contaminants in sediments and the emergence of toxicopathic effects in sole juveniles based on chemical analysis, biomarkers and histopathological approaches.

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Chapter III

Waterborne cadmium toxicopathic effects on sole, *Solea senegalensis*, juveniles

Congress

Briaudeau T., Alves Dos Santos A., Zorita I., Izagirre U., Marigómez I. A multi-organ histopathological approach in juvenile *Solea senegalensis* exposed to model contaminants in laboratory conditions: cadmium and benzo[a]pyrene. 11th Iberian and 8th Iberoamerican Congress on Environmental Contamination and Toxicology (CICTA), Madrid, Spain, 11-13 July 2018. Platform presentation (T. Briaudeau).

Briaudeau T., Alves Dos Santos A., Guerrero-Limón G., Marigómez I., Zorita I., Izagirre U. Biochemical biomarkers and histopathology in juvenile *Solea senegalensis* for early warning assessment of marine ecosystem health. SETAC Europe 28th annual meeting, Rome, Italy, 13-17 May 2018. Poster presentation.

Abstract

Estuarine areas experiencing anthropogenic pressure may receive a wide variety of contaminants present as a mixture. The bioavailability of each chemical varies according to the nature of the element, environmental conditions (pH, salinity, temperature) and sediment characteristics (granulometry, redox potential), making the assessment of chemical toxicity complicated. In laboratory conditions, the use of single contaminant for toxicity assays allows for the clarification of biological responses to environmental stressors. In the present investigation, *S. senegalensis* juveniles were exposed to different concentrations of waterborne Cd. Sole juveniles (n=13 per group) were retrieved after 3 and 7 d exposure. Brain, liver, gills and gonads were dissected out and processed to determine biomarkers of neurotoxicity and oxidative stress, lysosomal biomarkers and histopathology. Additional liver and muscle samples were collected for chemical analysis at day 7. Biological responses were consistent with waterborne Cd concentration and exposure time. Brain acetyl cholinesterase was inhibited, and liver catalase and glutathione-*S*-transferase were first induced and then inhibited in a dose dependent manner. A dose dependent lysosomal membrane destabilisation was more pronounced at day 7. Histopathological lesions in gills, liver and gonad were more frequent at day 7 in soles exposed to high Cd concentrations. In agreement, the Integrative Biological Response index (IBR/n) indicated a dose-dependent decline in health condition upon exposure to waterborne Cd ($IBR/n_{High\ Cd} > IBR/n_{Mid\ Cd} > IBR/n_{Low\ Cd} > IBR/n_{Control}$). The present investigation evidenced toxicopathic effects of waterborne Cd in sole juveniles and supports the use of biomarkers and histopathology approaches as early-warning indicators of altered health status in sole.

Résumé

Les zones estuaires sous forte pression anthropique sont susceptibles de recevoir une grande variété de polluants sous forme de mélange. La biodisponibilité de chaque contaminant varie en fonction de la nature de l'élément, de facteurs environnementaux (pH, salinité, température) et des charactéristiques du sédiment (granulométrie, potentiel redox), ce qui complique l'évaluation de la toxicité chimique. En laboratoire, l'utilisation de contaminants isolés pour des tests de toxicité permet de clarifier les effets biologiques de la contamination environmentale. Dans cette expérience, des soles juvéniles ont été exposées à différentes concentrations de Cd. Des échantillons de cerveau, foie, branchies et gonades ont été prélevés dans chaque groupe expériemental (n=13) après 3 et 7 jours d'exposition au métal afin d'y analyser des biomarqueurs de neurotoxicité, de stress oxidatif et de lysosomes, ainsi que l'histopathologie des branchies, du foie et des gonades. Des échantillons de foie et de muscle ont également été prélevés pour des analyses chimiques. Globalement, les réponses biologiques étaient cohérentes avec les niveaux de contaminant détectés dans l'eau et la durée d'exposition. L'inhibition enzymatique de l'acétylcholinestérase, enzyme du cerveau, a révélé des effets neurotoxiques évidents du Cd. La catalase et la glutathion-*S*-transférase du foie étaient induites après 3 jours d'exposition au Cd mais inhibées après 7 jours, en particulier dans le cas de fortes concentrations. De même, la déstabilisation de la membrane lysosomale était proportionnelle à la concentration en contaminant, notamment après 7 jours. Les lésions histopathologiques identifiées dans les branchies, le foie et les gonades étaient plus fréquentes après 7 jours d'exposition au Cd, en particulier à plus fortes concentrations. L'intégration des réponses biologiques sous forme de l'index « Integrative Biological Response index » (IBR/n) a permis de détecter une diminution de l'état de santé des soles en relation avec les concentrations de Cd appliquées ($IBR/n_{High\ Cd} > IBR/n_{Mid\ Cd} > IBR/n_{Low\ Cd} > IBR/n_{Control}$). La recherche suivante met en évidence les effets toxicopathologiques du Cd chez la sole juvénile et soutient l'utilisation de biomarqueurs et de l'histopathologie comme indicateurs rapides d'altérations d'état de santé de la sole face au stress environnemental.

Laburpena

Presio antropogenikoa jasaten duten estuarioek kutsatzaile desberdinak jaso ditzakete nahaste moduan. Elementu kimiko bakoitzaren bioeskuragarritasuna, elementuaren izaeraren, ingurumen baldintzen (pH-a, gazitasuna, tenperatura) eta sedimentuaren ezaugarrien (granulometria, erredox potentziala) arabera aldatzen da, toxizitate kimikoaren ebaluazioa zailduz. Laborategi baldintzetan, toxizitate esperimentuetan banakako kutsatzaileen aurkako erantzun biologikoak argitzea ahalbidetzen du. Ikerketa honetan, *S. senegalensis* gazteak uretan Cd kontzentrazio desberdinetara esposatuak izan ziren. Mihi-arrain gazteak (n=13 taldeko) 3 eta 7 esposizio egunen ostean jasoak izan ziren. Garun, gibel, zakatz eta gonadak disekzionatuak eta prozesatuak izan ziren neurotoxizitate eta estres oxidatiboko biomarkatzaileak, biomarkatzaile lisosomikoak eta histopatologia determinatzeko. Gibel eta muskulu lagin gehigarriak jaso ziren analisi kimikoetarako 7. egunean. Erantzun biologikoak uretako Cd kontzentrazio eta esposizio denborarekiko koherenteak izan ziren. Garuneko azetilkolinisterasa inhibitu zen, eta gibelean katalasa eta glutationa-S-transferasa lehenik induzitu ziren eta ondoren inhibitu ziren Cd dosiaren menpe. Dosiaren araberako mintz lisosomikoaren desestabilizazioa esanguratsua ikusi zen 7. egunean. Lesio histopatologikoak zakatz, gibel eta gonadetan sarriagoak izan ziren 7. egunean Cd kontzentrazio altuetara esposatutako mihi-arrainetan. Honekin guztiarekin bat egin zuen “Integrative Biological Response Index (IBR/n)” indizeak, uretako Cd esposizioaren araberako osasun egoeraren beherakada adieraziz ($IBR/n_{Altua\ Cd} > IBR/n_{Ertaina\ Cd} > IBR/n_{Baxua\ Cd} > IBR/n_{Kontrola}$). Ikerketa honek, uretako Cd esposizioak mihi-arrain jubeniletan efektu toxikopatikoak dituela frogatu du eta baita biomarkatzaile zein histopatologiaren hurbilketak arrainen osasun egoeraren aldaketak antzemateko adierazle goiztiarrak direla babestu ere.

1. Introduction

Estuarine ecosystems under anthropogenic pressure are exposed to a wide variety of contaminants present as a mixture. Chemicals can be found dissolved in water or accumulated in the sediment where they can stay trapped for extended periods (Pan and Wang, 2012). Nevertheless, sediment contaminants can also be released back to the overlying water and become potentially toxic for the resident benthos (Eggleton and Thomas, 2004). Thus, the association between the environmental levels of chemicals and alterations in the general health status recorded in the biota is intricate, and laboratory assays using model contaminants under controlled conditions are essential to establish this association.

Amongst benthic species, sole juveniles and adults are used in the field as sentinels for the environmental risk assessment of estuarine ecosystems (Claireaux et al., 2004; Oliva et al., 2010, 2012a, 2012b, 2013, 2014; Gonçalves et al., 2013, 2014; Solé et al., 2013, 2016; Cuevas et al., 2015a, 2015b; Siscar et al., 2015; Chapter 1). Likewise, changes in general health status elicited by pollutants have been investigated in sole under laboratory conditions both in sediment (Riba et al., 2004; Costa and Costa, 2008, Costa et al., 2009a, 2009b; Ribecco et al., 2012; Ghribi et al., 2019; Chapter 2) and waterborne exposure experiments (Claireaux and Davoodi, 2002; Claireaux et al., 2004; Salamanca et al., 2008; Kalman et al., 2010; López-Galindo et al., 2010a, 2010b; Costa et al., 2013; Sànchez-Nogué et al., 2013; Martins et al., 2015).

Heavy metals are contaminants of major concern; they have attracted great interest in toxicological studies due to their abundance, their persistence and their toxicity (Pan and Wang, 2012; Islam et al., 2015). Amongst them, Cd is known to be highly toxic leading to genotoxic effects as well as to severe metabolic, cellular and tissue-level damage (Hallare et al., 2005; Costa and Costa, 2008; Levit, 2010). Thus, it has been used in laboratory experiments as model contaminant to understand bioaccumulation and toxicity of metals (Pan and Wang, 2012; Pereira et al., 2016). Biological responses to Cd exposure include biochemical, cellular and tissue-level biomarkers (UNEP/RAMOGE, 1999; Davies and Vethaak, 2012; OSPAR Commission, 2013).

Acetylcholinesterase (AChE) is an enzyme involved in the degradation of neurotransmitters (Méndez-Armenta and Ríos, 2007; Pretto et al., 2010). Changes in this enzyme activity in the brain are used in sole and other fish species as biomarkers of exposure to neurotoxic pollutants (Grue et al., 1997; Heath et al., 1997; Minier et al., 2000; López-Galindo et al., 2010a, 2010b; Davies and Vethaak, 2012; Oliva et al., 2012a; Solé et al., 2012; Jebali et al., 2013; Siscar et al., 2013; Burgeot et al., 2017). Amongst them, Cd and other metals (Cu, Hg, and Zn) are known to inhibit brain AChE activity (Frasco et al., 2005; Davies and Vethaak, 2012). Metals can also produce oxidative stress (Méndez-Armenta and Ríos, 2007; Regoli et al., 2011; Alijani et al., 2017), which is reflected by enhanced production of reactive oxygen species (ROS), disturbance of antioxidant defences and alterations in xenobiotic metabolism (Di Giulio et al., 1989; Sies et al., 1991; Livingstone, 2001; Roméo et al., 2013; Regoli and Giuliani, 2014). In sole

and other fish species, changes in superoxide dismutase (SOD) and catalase (CAT) activities in the liver were related to the presence of Cd, both in metal contaminated sites and upon exposure in the laboratory (Atli et al., 2006; Oliva et al., 2012b; Souid et al., 2013). On the other hand, the activity of hepatic glutathione-*S*-transferase (GST) is enhanced in sole exposed to metals and other pollutants in mixtures (Fonseca et al., 2011a; 2011b; Ghribi et al., 2019).

Lysosomal alterations are effect biomarkers used in fish as non-specific responses to a variety of environmental stressors (Köhler, 1991; Heath, 1995; Hinton et al., 2001; Köhler et al., 2002; JAMP, 2003; van der Oost et al., 2003; Au, 2004; Baršienė et al., 2006; ICES, 2006; Zorita et al., 2008; Davies and Vethaak, 2012; Burgeot et al., 2017). The lysosomal membrane integrity is a core biomarker in biological effects assessment (Law et al., 2010; Davies and Vethaak, 2012). Typically, lysosomal enlargement, membrane destabilisation and changes in lysosomal content were recorded in fish collected from polluted sites (Broeg et al., 2002, 2005; Köhler et al., 2002; Einsporn et al., 2005; Baršienė et al., 2006; Zorita et al., 2008; Burgeot et al., 2017). Lysosomal changes have been reported upon Cd exposure in laboratory studies for molluscs (Viarengo et al., 1987; Marigómez et al., 1989, 2005; Lekube et al., 2000; Izagirre et al., 2014) and fish species, including sole (Alvarado et al., 2005; Le Croizier et al., 2019).

Gill and liver histopathology are considered powerful indicators of medium and long-term effects of exposure to pollutants in flatfish, including sole (Myers et al., 1994; Bernet et al., 1999; Stentiford et al., 2003; Feist et al., 2004; Salamanca et al., 2008; Costa et al., 2009a; Gonçalves et al., 2013). Gonad histo(patho)logy is also essential to survey the reproduction status of a population and to assess potential tissue-level lesions, both indicative of the liability of the future generations (Blazer, 2002; Solé et al., 2016). Multi-organ histopathological approaches have been applied in sole in field and laboratory studies (Jímenez-Tenorio et al., 2008; Costa et al., 2009b; Oliva et al., 2013; Cuevas et al., 2015a, 2015b; Chapter 1). Cadmium toxicity has been reported based on fish histopathology (Rani and Ramamurthi, 1989; van Dyk et al., 2007; Kumar and Singh, 2010) and in particular in sole (Costa et al., 2010, 2013).

A previous experiment demonstrated that sediment toxicity in sole was related to the transfer of contaminants from the sediment to the water column, in particular for metals (Chapter 2). Thus, the present investigation aimed at identifying toxicopathic effects of waterborne metal contamination in juvenile *Solea senegalensis* upon laboratory exposure, using Cd as a model of metal toxicity. Biomarkers of oxidative stress and neurotoxicity, lysosomal biomarkers and histopathology were determined after 3 and 7 d exposure. All these biomarkers can be synthetized into the Integrative Biological Response (IBR) index (Beliaeff and Burgeot, 2002; Broeg et al., 2005; Marigómez et al., 2013).

2. Material and Methods

2.1 Experimental setup

Solea senegalensis juveniles (24.5±1.8 cm standard length; 165.0±37.1 g total wet-wt) were exposed to different concentrations of Cd (Control; Low Cd: 1 µg/l; Mid Cd: 10 µg/l and High Cd: 1000 µg/l) for 7 d at optimal stocking density (4-6 kg/m; Schram et al., 2006). Each experimental group was placed in a closed-system and water changes were performed every two days to ensure optimal water conditions: pH=8, salinity=31-33 PSU, temperature=13-14 ºC, dissolved O_2=6-8 mg/l and total ammonia=0 mg/l. Photoperiod throughout the experiment was set at 12:12 h light:dark. Fish were daily fed with commercial food (0.3g per fish; BioMar Iberia S.A., Dueñas, Spain).

2.2 Chemical analysis of water samples

Water samples were collected from each experimental group 3, 24 and 48 hours after contaminant load. Analyses of Cd content in water (1:10, v/v) were performed by inductively coupled plasma with mass detector (7700x, Agilent Technologies, Palo Alto, USA) using a MicroMist micro-uptake glass concentric nebulizer (Glass Expansion, West Melbourne, Victoria, Australia). In order to reduce MO^+ formation in the plasma, the spray chamber was Peltier cooled at 2ºC. Finally, standard nickel cones (sample and skimmer) were generally used. The acquisition masses and integration times provided more than sufficient sensitivity to meet all certified values. The optimization of the ICP-MS conditions was achieved by adjusting the torch position and tuning for reduced oxide and doubly charged ion formation with a standard tuning solution containing 1.0 µg/l of 7Li, 24Mg, 59Co, 89Y, 140Ce and 205Tl in 1.0% HNO_3. This equipment includes a collision cell (He gas, ORS3 system, Agilent Technologies ©) for discriminate spectral interferences with high performance for all the trace metals considered in here. In addition, EPA 6020 recommendations were followed for interference overcoming such as correction equations for cadmium.

2.3 Fish biometry

Individual wet-wt (W in g) and length (L in cm) and liver and gonad wet-wt (LW and GW in g, respectively) were recorded to calculate (a) $K=W\times100/L^3$; (b) HSI= LW×100/W; and (c) GSI=GW×100/W; where K is the condition factor, HSI is the hepatosomatic index, and GSI is the gonadosomatic index. *Analysis of Cd levels in liver and muscle*

Liver and muscle samples were collected at day 7 for chemical analysis. Liver and muscle tissue from 6 individuals per treatment were pooled to obtain a minimum of 1 g dw. After lyophilisation, tissue samples were digested in acid (HNO_3) at 180ºC for 15min, using a microwave system (MARS 5 Xpress CEM Corporation Instrument). Cadmium content was determined by inductively coupled plasma with mass detector (7700x, Agilent

Technologies, Palo Alto, USA) using a MicroMist micro-uptake glass concentric nebulizer (Glass Expansion, West Melbourne, Victoria, Australia).

2.4 Biochemical analysis

At days 3 and 7 of exposure, liver and brain samples were dissected out, rapidly frozen and maintained at -80ºC until use. Samples were processed for biochemical analysis; they were homogenised (1:4 for liver and 1:5 for brain) in 0.1 M potassium phosphate buffer (pH 7.4) and centrifuged for 30 min at 12000 *g* at 4ºC to obtain the post-mitochondrial supernatant (PMS). Catalase (CAT), superoxide dismutase (SOD) and glutathione-*S*-transferase (GST) enzyme activities were determined in liver PMS and acetylcholinesterase (AChE) activity in brain PMS using a BioTek Eon microplate spectrophotometer. Enzyme activities were expressed as a function of the protein concentration in the samples. Total protein content in the homogenates was measured in triplicate at 595 nm following Bradford's method adapted to microplate and using bovine serum albumin as standard (Bradford, 1976; Guilhermino et al., 1996). All enzyme assays were performed at 25ºC.

Catalase (CAT). CAT activity was determined by the method of Claiborne (1985) by measuring the rate of enzyme decomposition of hydrogen peroxide (H_2O_2) determined as absorbance decrease at 240 nm. The reaction medium (final volume of 10 ml) contained 9977 µl of 50 mM phosphate buffer (pH 7.0) and 23 µl of hydrogen peroxide (H_2O_2; 30% v/v). The reaction was started by the addition of 5 µl of samples to 295 µl of reaction medium. Absorbance decrement was measured for 3 min at 240 nm. Results were expressed as µmol H_2O_2/min/mg protein.

Superoxide dismutase (SOD). SOD activity was determined by a colorimetric method using a SIGMA kit (SOD Determination kit; ref: SIGMA 19160) to measure the superoxide anion reduction as proportional to the SOD inhibition activity. Each well contained 200 µl of WST (water soluble tetrazolium salt) working solution, 20 µl of enzyme working solution and 20 µl of sample and were left incubating at 37ºC for 20 min. Three different blanks were prepared for the assay: Blank 1 (200 µl of WST working solution, 20 µl of enzyme working solution and 20 µl of ultrapure water); Blank 2 (200 µl of WST working solution, 20 µl of dilution buffer and 20 µl of sample); and Blank3 (200 µl of WST working solution, 20 µl of dilution buffer and 20 µl of ultrapure water). Absorbance was measured at 450 nm and SOD activity (inhibition rate %) was calculated as follow:

$$\text{SOD activity (inhibition rate \%)} = \frac{(A1-A3)-(AS-A2)}{(A1-A3)} \times 100;$$

where A1 is the absorbance of Blank 1, A2 is the absorbance of Blank 2, A3 is the absorbance of Blank 3 and AS is the absorbance of the samples.

Glutathione-S-transferase (GST). GST activity was determined by the Habig's method (Habig et al., 1974) adapted to microplate and using bovine serum albumin as standard

(Guilhermino et al., 1996). Enzyme activity was measured following the formation of thioether by conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB). The reaction medium contained 9.9 ml of 100 mM potassium phosphate buffer (pH 7.4), 1.8 ml of 10 mM GSH solution and 300 µl of 60 mM CDNB solution. Each well contained 100 µl of samples and 200 µl of reaction medium. Enzyme activity was measured at 340 nm for 6 min and expressed as nmol/min/mg protein.

Acetylcholinesterase (AChE). AChE activity was determined according to the Ellman 's colorimetric method of (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996) by measuring the formation of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) at 412 nm. The reaction medium contained 200 µl of 75 mM acetylcholine solution, 1 ml of 10 mM DTNB and 30 ml of 100 mM potassium phosphate buffer (pH 7.4). Each well contained 50 µl of samples and 250 µl of reaction medium. After 10 min of incubation, enzyme activity was recorded over 10 min. AChE activity was expressed as specific activity (nmol DTNB/min/mg protein).

2.5 Lysosomal biomarkers

At days 3 and 7 of exposure, liver samples were dissected out, rapidly frozen and maintained at -80ºC until use. Frozen samples were processed using Tissue Array (TA) technology (Array Mold® Kit; n°20015-A) and TA blocks were cut at -27ºC using a Leica CM 3050S cryotome.

Lysosomal membrane stability (LMS). The determination of lysosomal membrane stability was based on the time of acid labilisation treatment required to produce the maximum staining intensity according to UNEP/RAMOGE (1999), after demonstration of acid phosphatase (AcP) activity in hepatocyte lysosomes. Ten serial cryotome sections (10 µm) were subject to acid labilisation in intervals of 0, 2, 4, 6, 8, 10, 15, 20, 25, 30, 40 and 50 min in 0.1 M citrate buffer (pH 4.5, containing 2.5% NaCl) in a shaking water bath at 37ºC. The demonstration of AcP activity was performed by incubation of the sections in a substrate incubation medium (naphthol AS-BI-phosphate, dimethylsulfoxide, 0.1 M citrate buffer at pH 4.5, containing 2.5% NaCl and low viscosity polypeptide, Polypep®) for 20 min at 37ºC, in a shaking bath. Rinsed sections (3% NaCl at 37ºC for 5 min) were stained at room temperature with diazonium dye Fast Violet B salt (1 mg/ml in 0.1 M phosphate buffer, pH 7.4) for 9 min. Slides were fixed in Baker's formol calcium containing 2.5% NaCl for 10 min at 4ºC, rinsed in distilled water and mounted in Kaiser´s glycerine gelatine.

The time of acid labilisation treatment required to produce the maximum staining intensity was assessed under a light microscope as the maximal accumulation of reaction product associated with lysosomes (UNEP/RAMOGE, 1999) and was denoted as the Labilisation Period (LP; in min). Four determinations were made per individual; for each area, the first maximum staining peak was considered to determine the LP value (ICES, 2015). A final LP value was calculated for each individual fish as the mean of the four LP values determined in each area.

Lysosomal Structural Changes (LSC). The determination of changes in the size and numbers of lysosomes was made according to the method described by Cajaraville et al. (1989) for mussels, further on adapted to fish liver by Alvarado et al. (2005), after histochemical demonstration of β-glucuronidase activity in fish hepatocytes. Cryotome sections (8 μm) were incubated in freshly prepared β-glucuronidase substrate incubation medium (naphthol AS-BI-β-glucuronidep, 50 mM sodium bicarbonate, 0.1 M acetate buffer at pH 4.5, containing 2.5% NaCl and polyvinyl alcohol at 15%) for 20 min at 37ºC. Slides were rinsed (2.5% NaCl at 37ºC for 2 min) and transferred to a postcoupling medium (Fast Garnet, 0.1 M phosphate buffer at pH 7.4 containing 2.5% NaCl) for 10 min at room temperature, in the dark. Sections were fixed in Baker's formol calcium solution containing 2.5% NaCl for 10 min at 4 ºC, rinsed in distilled water and mounted Kaiser's glycerol gelatine.

The structure of lysosomes was assessed through a stereological procedure based on image analysis (BMS, Sevisan) according to Cajaraville et al. (1991). Five measurements using a 100× objective lens were made per individual. The mean value of the following stereological parameters was determined for the lysosomes of each liver sample (Lowe et al., 1981): volume density ($Vv_L=V_L/V_C$), surface density ($Sv_L=S_L/V_C$), surface-to-volume ratio ($S/V_L=S_L/V_L$) and numerical density ($Nv_L=N_L/V_C$); where V=volume, S=surface, N=number, L=lysosomes and C=liver cytoplasm.

Intracellular accumulation of neutral lipids. Changes in levels of neutral lipids were determined according to Marigómez and Baybay-Villacorta (2003), after Oil Red O (ORO) staining to visualise neutral lipids (Culling, 1974). Cryotome sections (8 μm) were fixed in Baker's formol calcium containing 2.5% NaCl for 15 min at 4ºC. Air dried sections were washed in isopropanol (60%) and stained with ORO for 20 min. The staining solution (stable for 1-2 h) was freshly made from a saturated stock ORO solution (0.3% in isopropanol) and kept protected from the light. Stained sections were differentiated in 60% isopropanol, rinsed in water, counterstained with 1% Fast Green FCF for 20 min and mounted in Kaiser's glycerine.

Five measurements using a 40× objective lens were made per individual. The mean value of the volume density ($Vv_{NL}=V_{NL}/V_C$) of neutral lipids was determined; where V=volume, NL=neutral lipids and C=liver cytoplasm.

2.6 Histological processing and histopathological examination

At days 7 and 28 of exposure, gill, liver and gonad samples were dissected out (n=12 per experimental group). Gills were fixed in Bouin's solution for 24 h at 4ºC and rinsed in formic acid (8% v/v) for 24 h at room temperature. Liver and gonad samples were fixed in 4% neutral buffered formol for 24 h at 4ºC. Fixed samples were dehydrated in a graded series of ethanol, cleared and embedded in paraffin (Leica ASP 300S). A minimum of two sections (5 μm) per sample were obtained using a rotary microtome (Leica RM 2125RTS) and were stained with hematoxylin-eosin (H&E; Martoja and Martoja-Pierson, 1970).

Histopathological examination. The examination of histological samples was made under a light microscope (Nikon Eclipse E200) starting with a 4× objective lens for a general description of the organs. Higher power objective lenses (10×, 20×, 40× and 100×) were used for the identification of histopathological lesions.

Hepatic samples were analysed for histopathology based on the recommendations provided by ICES (1997) and the Biological Effects Quality Assurance in Monitoring Programmes (BEQUALM, 2001). The publications by Costa et al. (2009b) on liver and gill histopathology of wild sole juveniles, Zorita and Cuevas (2014) on hepatic lesions commonly recorded in sole and Blazer (2002) on gonad histopathological lesions were used as guidelines to identify lesions in the particular case of sole. Amongst lesions identified, only persistent cases were considered for the calculation of histopathological indices and point alterations were discounted.

Histopathological indices. The semi-quantitative histopathological approach in liver was performed following two different methods.

The first approach was based on the scoring system proposed by Lang et al. (2006) for hepatic histopathology. For this purpose, hepatic lesions were classified into five categories as presented by Feist et al. (2004): (1) non-specific lesions; (2) early non-neoplastic toxicopathic lesions; (3) foci of cellular alteration (FCA); (4) benign neoplasms; and (5) malignant neoplasms. The stage (S) of each lesions recorded was determined as mild, medium and severe, depending on the size of the tissue area affected in the sections and the degree of cellular change observed. Lang's scoring system consisting of 15 lesion scores was used for the assessment of spatial and temporal variation in the lesions recorded. Lesions' scores were determined based on the lesion category and the lesion stage (S). If more than one lesion category was recorded in one specimen, the highest lesion score was used for assessment purposes. From the individual scores, mean histopathology liver lesion scores (LS_{liver}) were calculated for each sampling station and time.

The second semi-quantitative approach was based on the weighted histopathological index developed by Bernet et al. (1999). Accordingly, hepatic lesions were classified into five categories based on their reaction pattern: (1) circulatory disturbances; (2) inflammatory responses; (3) regressive changes; (4) progressive changes; and (5) tumours (neoplams). Each alteration was assigned an importance factor (w) as: (1) minimal pathological importance (the lesion is reversible after the cessation of pollutant exposure); (2) moderate pathological importance (the lesion is reversible in most cases if the exposure ends); (3) marked pathological importance (the lesion is generally irreversible and may lead to partial or total loss of organ function). The stage (a) of each lesion identified was ranked in 4 categories (0, 2, 4 and 6) according to the level of dissemination of the alteration in the organ; where 0 is absence and 6 is high degree of dissemination depending on the size of the tissue area affected in the sections and the degree of cellular change observed. Different histopathological indices were calculated using the lesion importance factor (w) and the lesion stage (a):

- the organ index $I_{org.}$ was calculated for each individual and for each organ as follow:

$$I_{org} = \Sigma_{rp} \Sigma_{alt} (w_{org\ rp\ alt} \times a_{org\ rp\ alt})$$

- the reaction index of an organ $I_{org\ rp}$ was calculated for each individual, each organ and each lesion category:

$$I_{org\ rp} = \Sigma_{alt} (w_{org\ rp\ alt} \times a_{org\ rp\ alt})$$

Gills and gonads lesions were also classified according to Bernet et al. (1999). Only I_{liver} and I_{gills} were used to calculate the total histopathological indices integrating the histopathological results from different organs:

- the total index I_{tot} was calculated for each individual, for all organs:

$$I_{tot} = \Sigma_{org} \Sigma_{rp} \Sigma_{alt} (w_{org\ rp\ alt} \times a_{org\ rp\ alt})$$

- the total reaction index I_{rp} was calculated for each individual and each lesion category, for all organs:

$$I_{rp} = \Sigma_{org} \Sigma_{alt} (w_{org\ rp\ alt} \times a_{org\ rp\ alt})$$

Histopathological indices. The prevalence of each histopathological alteration was determined as the percentage occurrence of an alteration within each experimental group for gills and liver and within each combination of experimental group and gender for gonads.

Characterisation of the reproductive cycle. Gonad histological sections were analysed at a light microscope for gender and gamete developmental stages determination. Male gamete developmental stages were determined according to García-López et al. (2006) and were classified in five stages as follow: Stage I (early spermatogenesis); Stage II (mid spermatogenesis); Stage III (late spermatogenesis); Stage IV (mature); Stage V (recovery). The identification of gamete developmental stages for females was mainly based on Murua and Motos (2006). Stages were classified as followed: Stage I (growth); Stage II (early vitellogenesis); Stage III (late vitellogenesis); Stage IV (maturation).

2.7 Integrative Biological Response (IBR/n) index

The IBR index (Beliaeff and Burgeot, 2002) was calculated based on the integration of biochemical (AChE, GST), histochemical (LP) biomarkers and hepatic histopathology (I_{gills}, I_{liver}) following the calculation method described by Marigómez et al. (2013). The calculation method is based on relative differences between the biomarkers in each given data set. Thus, the IBR index is computed by summing-up triangular starplot areas (multivariate graphic method) for each two neighbouring biomarkers in a given data set, according to the following procedure (Beliaeff and Burgeot, 2002; Devin et al., 2014): (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'=standardized 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 standardized value obtained for each sample to the absolute

standardized value of the minimum value in the data set: $y_i=x_i´ + |x_{min}´|$; (4) calculation of the Star Plot triangular areas as $A_i=(y_i \times y_{i+1} \times \sin\alpha)/2$, where y_i and y_{i+1} are the standardized values of each biomarker and its next biomarker in the star plot, respectively, and α is the angle (in radians) formed by each two consecutive axis where the biomarkers are represented in the Start Plot ($\alpha=2\pi/n$; where n is the number of biomarkers); and (5) calculation of the IBR index which is the summing-up of all the Star Plot triangular areas ($IBR=\Sigma A_i$).Since the IBR value is directly dependent on the number of biomarkers in the data set, the obtained IBR value must be divided by the number of biomarkers used (IBR/n; Broeg and Lehtonen, 2006).

In the present work, five biomarkers were integrated for the calculation of the index as IBR/n. Parameters were selected to represent effects of waterborne Cd at different biological organisation levels where biochemical and histochemical parameters demonstrate sub-cellular effects of contaminants and gill and liver histopathology indicate subsequent tissue-level effects.

2.8 Statistical analysis

Statistical analyses were carried out using IBM SPSS Statistics Base 22.0. Homogeneity of variance (Levene´s test) and normality of data (Shapiro's test) were tested before statistical analysis. Two-way ANOVAs were performed to analyse the effects of the wateborne concentration of Cd ($[Cd]_{water}$), exposure time (T) and their combination (Cd×T) on biomarkers and histopathology. Logarithmic transformation was applied to non-parametric variables (CAT, Nv_L and Vv_{NL}). For normal data, differences between experimental groups and throughout exposure time were tested using the parametric one-way Anova test and the T Student test, respectively. For non-normal data set, the non-parametric Kruskal-Wallis test and Mann-Whithney U test were used to analyse differences in biological data between experimental groups and throughout exposure time. The z-score test and the Pearson's Chi test were used to determine significant differences in histopathological lesions prevalence between experimental groups and throughout exposure time. Significant differences in chemical data were tested with the z-score test. The parametric Pearson's correlation test was used to assess associations between concentrations of Cd in water and in liver samples. Level of significance for all analyses was $p = 0.05$.

3. Results

3.1. Contaminant levels in water and biological samples

The concentrations of Cd measured in water are in agreement with the gradient of contaminant originally applied to each exposure group, with the highest concentration measured in the High Cd group at each sampling time (3, 24 and 48 hr; Table 1). Likewise, Cd content measured in liver after 7 d exposure was higher in High Cd exposed soles than the other experimental groups (Table 1). Levels of Cd in liver were significantly and positively correlated with Cd concentrations recorded in water ($R=0.999$, $n=4$, $p<0.05$). No Cd was detected in muscle samples (Table 1).

Table 1. Cd concentrations (µg/l) determined in water samples after 3, 24, and 48 hr of contaminant load. Cd content (µg/g wet-wt) determined in pools of liver and muscle samples collected from each experimental group at day 7. Different letters indicate significant differences between experimental groups (z score, $p<0.05$).

	$[Cd]_{sw}$			$[Cd]_{liver}$	$[Cd]_{muscle}$
	H3	H24	H48		
Control	0.77 [a]	0.63 [a]	0.36 [a]	2.29 [a]	<0.01
Low Cd	1.40 [a]	1.82 [a]	1.23 [a]	2.43 [a]	<0.01
Mid Cd	7.98 [a]	7.82 [a]	8.36 [a]	2.36 [a]	<0.01
High Cd	773 [b]	720 [b]	789 [b]	4.48 [b]	<0.01

3.2. Biological responses and toxicopathic effects

In total, 104 individuals were used. Length (24.5±1.8 cm) and W (165.0±37.1 g) were constant throughout the experiment. Likewise, the indices K (1.1±0.1), HSI (1.0±0.3), GSI_{male} (0.08±0.10) and GSI_{female} (0.94±0.19) did not differ between exposure groups.

Brain AChE activity was significantly affected by Cd×T (2-way ANOVA; Table 2), varying from 88.50±4.12 nmol/min/mg prot in Low Cd exposed soles at day 7 to 66.19±2.85 nmol/min/mg prot in High Cd exposed soles at day 7 (Figure 1A). Thus, AChE activities tended to increase in Control and Low Cd exposed soles at day 7 whilst activities from High Cd exposed soles decreased at day 7 and were lower than levels detected in other exposure groups. Hepatic CAT activity was significantly affected by $[Cd]_{water}$ and Cd×T (2-way ANOVA; Table 2). The highest CAT activity, detected in High Cd exposed soles at day 3, decreased significantly at day 7, being lower than levels recorded in the other exposure groups (Figure 1B). Hepatic GST activity was affected by T and Cd×T (2-way ANOVA; Table 2). Thus, levels of GST activity detected at day 3

Parameter	**Residual d.f.**	**F(Cd_{sw})**	**F(T)**	**F(Cd_{sw}xT)**
AChE	39	0.431	0.041	6.694**
CAT	39	3.057*	2.255	7.296***
SOD	39	0.165	5.665*	1.189
GST	39	1.866	49.534***	3.561*
LP	76	53.150***	38.750***	50.628***
Vv_L	70	10.355***	1.498	6.722**
Nv_L	70	3.939*	1.232	3.010*
Vv_{NL}	70	4.307**	3.724	0.293
I_{Tot}	104	4.433**	4.770*	1.317
I_{gills}	104	4.772**	2.965	1.452
I_{liver}	104	3.445*	9.610**	1.146
Female I_{gonad}	54	7.855***	1.647	0.426

No significant effect of S, T or S×T was detected for S/V_L, gills, liver and gonad lesion stages, LS_{liver}, male Igon.

Table 2. Summary of the 2-way ANOVAs performed to analyse the effects of Cd (d.f.: 3), time of exposure (d.f.: 1) and their combination ("Cd × Time", d.f.: 3) on biomarkers and histopathology (lesion stages and indices) in *S. senegalensis* exposed to different concentrations of Cd for 3 and 7 days. Logarithmic transformation was applied to CAT, Nv_L and Vv_{NL} (non-parametric variables). d.f.: degrees of freedom; F: Fisher's F; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

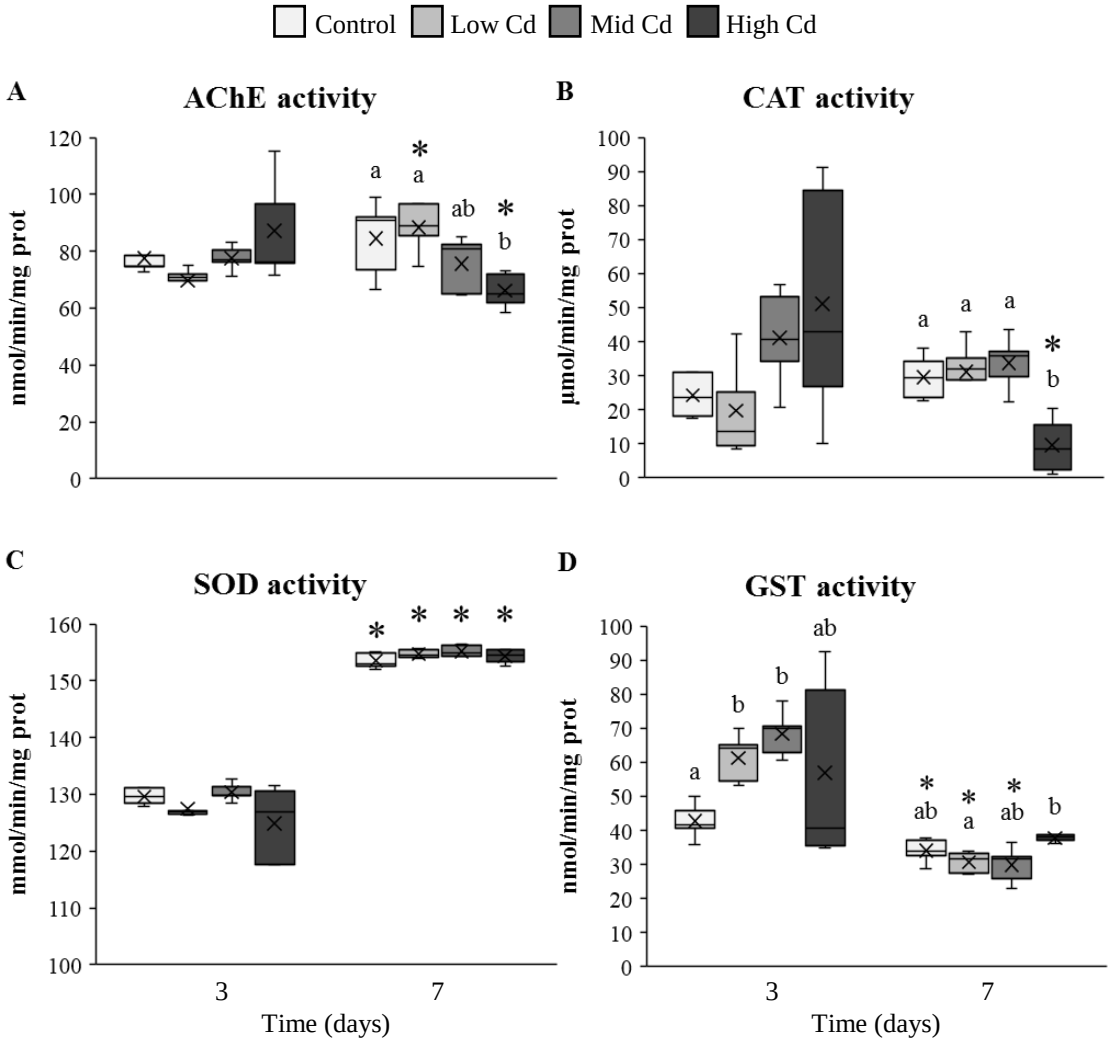


Figure 1. Brain acetylcholinesterase (A) and hepatic catalase (B), superoxide dismutase (C) and glutathione-*S*-transferase (D) enzyme activities measured in *S. senegalensis* exposed to different concentrations of Cd for 3 and 7 days. Different letters indicate significant differences between experimental groups of the same sampling time; asterisks indicate significant differences between exposure times ($p<0.05$); AChE: acetylcholinesterase; CAT: catalase; SOD: superoxide dismutase; GST: glutathione-*S*-transferase.

were higher in Cd exposed soles than in Control soles and decreased in all experimental groups at day 7 (Figure 1C). In contrast, hepatic SOD activity only varied with time (2-way ANOVA; Table 2), being higher at day 7 than at day 3 for all the exposure groups (Figure 1D).

Lysosomal LP was significantly affected by $[Cd]_{water}$, T and Cd×T (2-way ANOVA; Table 2); ranging from 30.48±1.64 min in Control soles at day 7 to 7.48±1.00 min in High Cd exposed soles at day 7 (Figure 2A). LP increased at day 7 in Control soles whilst it decreased in all Cd exposed groups, in particular in the case of Mid Cd and High Cd groups (Figure 2A). Lysosomal Vv_L and Nv_L were significantly affected by $[Cd]_{water}$ and Cd×T (2-way ANOVA; Table 2). At day 3, the lowest Vv_L was recorded in High Cd exposed soles whilst the values of other lysosomal biomarkers were constant between

exposure groups (Figure 2B-D). At day 7, the highest Vv_L and the lowest S/V_L were detected in Mid Cd exposed soles (Figure 2B-C) and Nv_L only decreased in Low Cd exposed soles (Figure 2D). Vv_{NL} was significantly affected by $[Cd]_{water}$ (2-way ANOVA; Table 2); ranging from 0.046±0.013 $\mu m^3/\mu m^3$ (High Cd soles, at day 7) to 0.252±0.106 $\mu m^3/\mu m^3$ (Low Cd soles, at day 3; Figure 2E). Thus, Vv_{NL} raised transiently in Low Cd exposed soles at day 3 and then returned to levels similar to those of the other experimental groups at day 7.

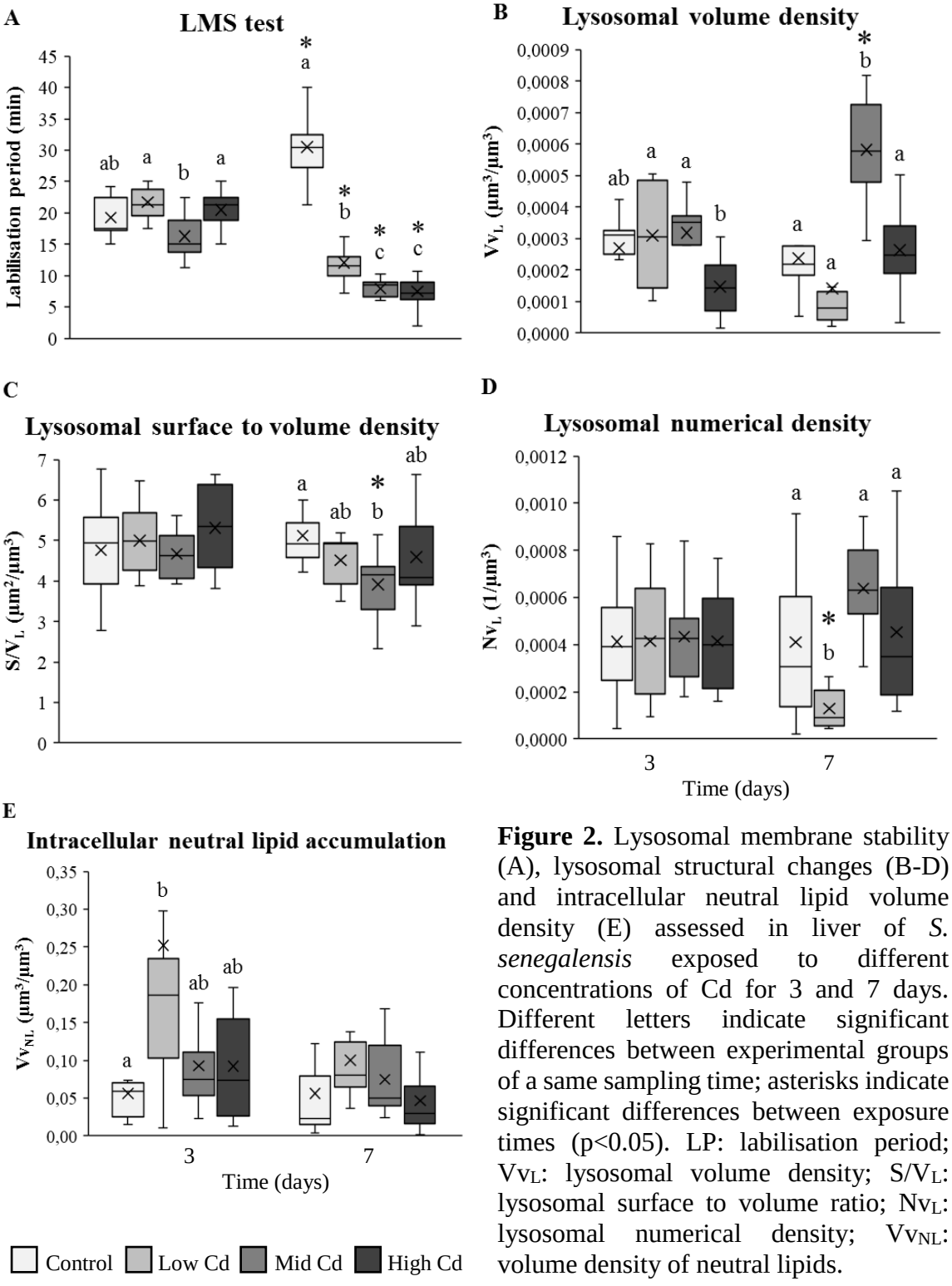


Figure 2. Lysosomal membrane stability (A), lysosomal structural changes (B-D) and intracellular neutral lipid volume density (E) assessed in liver of *S. senegalensis* exposed to different concentrations of Cd for 3 and 7 days. Different letters indicate significant differences between experimental groups of a same sampling time; asterisks indicate significant differences between exposure times ($p<0.05$). LP: labilisation period; Vv_L: lysosomal volume density; S/V_L: lysosomal surface to volume ratio; Nv_L: lysosomal numerical density; Vv_{NL}: volume density of neutral lipids.

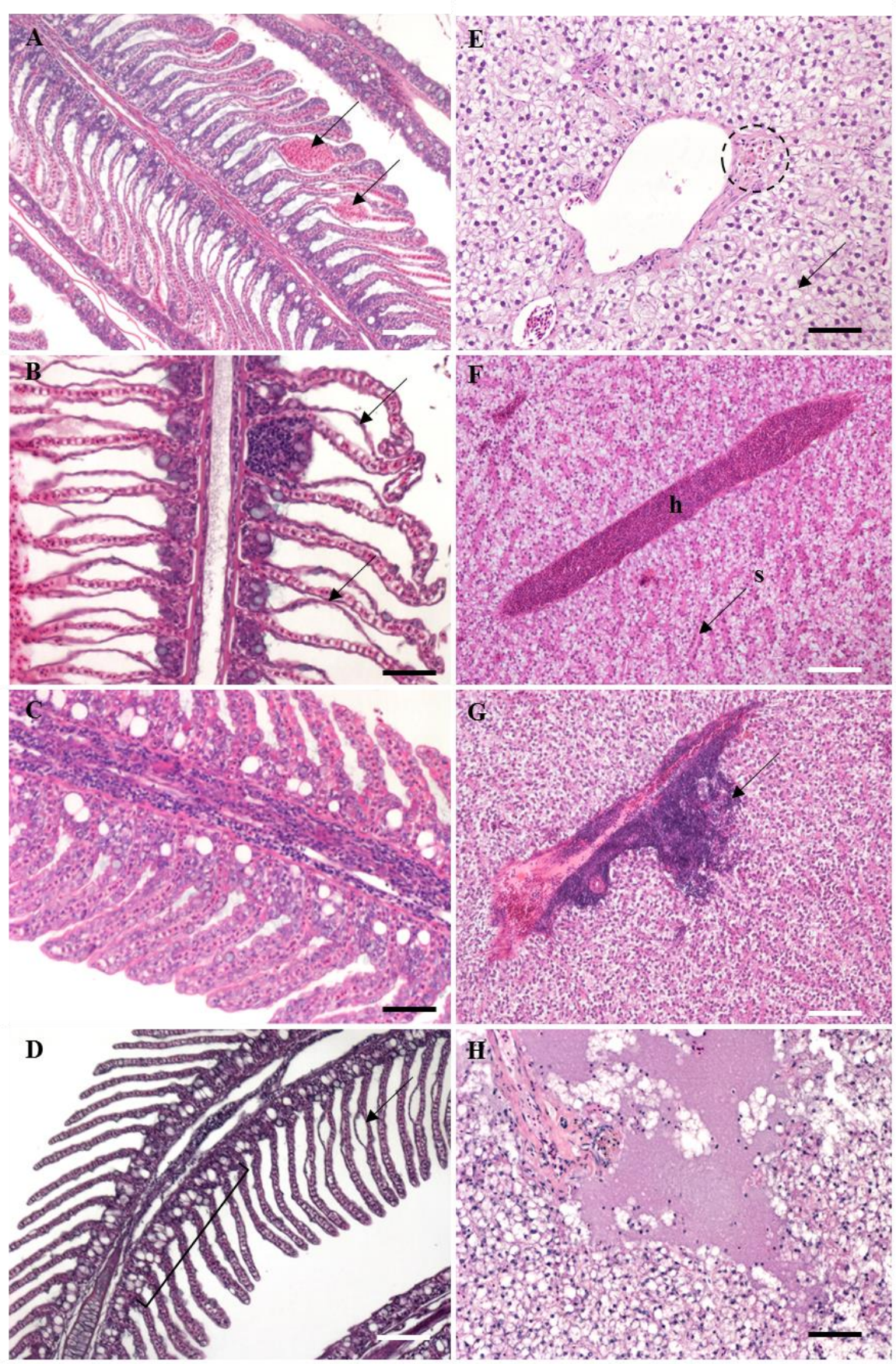

A
E
B
F
h
s
C
G
D
H

Table 3. Lesions prevalence (%) and means and standard errors of lesions stages (n=13; lesion intensity when detected) for gill histopathological lesions identified in *S. senegalensis* collected from each experimental group at days 3 and 7. Bold values indicate significant differences between experimental groups from the same sampling time ($p<0.05$); asterisks indicate significant differences between sampling days ($p<0.05$).

		Day3				Day7			
Gill lesions	***w***	**Control**	**Low Cd**	**Mid Cd**	**High Cd**	**Control**	**Low Cd**	**Mid Cd**	**High Cd**
Circulatory disturbances									
Lamellar capillary aneurysm	1	100.0 (2.5±0.2)	100.0 (2.5±0.2)	100.0 (2.9±0.3)	**92.3** (2.3±0.2)	92.3 (2.2±0.2)	100.0 (2.0±0.0)	92.3 (2.5±0.3)	100.0 (2.8±0.3)
Regressive changes									
Epithelial lifting	1	92.3 (2.3 ± 0.2^{a})	100.0 (2.2 ± 0.2^{a})	92.3 (2.8 ± 0.3^{ab})	100.0 (3.2 ± 0.3^{b})	76.9 (2.6±0.3)	76.9 (2.6±0.3)	84.6 (2.9±0.3)	**100.0** (2.6±0.3)
Progressive changes									
Hypertrophy of squamous epithelium	1	53.8 (2.3±0.3)	61.5 (2.3±0.3)	**69.2** (2.9±0.4)	**46.2** (2.0±0.0)	**23.1** (2.0±0.0)	76.9 (2.0±0.0)	69.2 (2.0±0.0*)	69.2 (2.0±0.0)
Chloride cell hyperplasia	2	46.2 (2.0±0.0)	38.5 (2.4±0.4)	**76.9** (2.2±0.2)	38.5 (2.4±0.4)	**23.1** (2.7±0.7)	38.5 (2.0±0.0)	61.5 (2.0±0.0)	**84.6*** (2.7±0.3)

w: lesion importance factor.

Gill histopathological lesions included lamellar capillary aneurysm (Figure 3A), epithelial lifting (Figure 3B), hypertrophy of squamous epithelium (Figure 3C) and chloride cell hyperplasia (Figure 3D). Overall, lesions were recorded at low lesion stages (Table 3). The high prevalence of lamellar capillary aneurysm and epithelial lifting did not vary between experimental groups (Table 3). In contrast, hypertrophy of squamous epithelium was more frequently recorded upon Cd exposure, in particular at day 7. At day 3, chloride cell hyperplasia was recorded at mild prevalence in all groups, except in Mid Cd exposed soles where prevalence was high. At day 7, the prevalence of this lesion increased with Cd concentration (Table 3). Yet, the prevalence of hypertrophy of squamous epithelium and chloride cell hyperplasia in the Control group was lower at day 7 than day 3. No tumours were detected in gills.

Figure 3. Histological sections (5 µm) of *S. senegalensis* exposed to different concentrations of Cd for 3 and 7 days, stained with hematoxylin-eosin. (A) Gill tissue showing capillary aneurysm (arrows); (B) severe case of gill epithelial lifting (arrows); (C) high degree of hypertrophy of squamous epithelium; (D) gill section showing a case of chloride cell hyperplasia (segment) with slight epithelial lifting (arrow); (E) hepatic tissue containing melanomacrophage centres (dotted circle) and fat vacuolation (arrow); (F) hepatic hyperaemia with accumulation of erythrocytes in blood vessels; h: hyperaemia; s: sinusoid; (G) presence of a large lymphocytic infiltration in liver (arrow); (H) severe case of hepatic necrosis. Black scale bar: 50 µm; white scale bar: 100 µm.

Table 4. Lesions prevalence (%) and means and standard errors of lesions stages (n=13; lesion intensity when detected) for liver histopathological lesions identified in *S. senegalensis* collected from each experimental group at days 3 and 7. Bold values indicate significant differences between experimental groups from the same sampling time ($p<0.05$); asterisks indicate significant differences between sampling days ($p<0.05$).

		Day3				Day7			
Hepatic lesions	***w***	**Control**	**Low Cd**	**Mid Cd**	**High Cd**	**Control**	**Low Cd**	**Mid Cd**	**High Cd**
Circulatory disturbances									
Haemorrhage	1	7.7 (2.0±0.0)	7.7 (2.0±0.0)	7.7 (2.0±0.0)	7.7 (2.0±0.0)	**0.0** (0.0)	30.8 (2.0±0.0)	15.4 (2.0±0.0)	30.8 (2.0±0.0)
Hyperaemia	1	**23.1** (2.0±0.0)	7.7 (2.0±0.0)	**0.0** (2.0±0.0)	15.4 (2.0±0.0)	7.7 (2.0±0.0)	0.0 (2.0±0.0)	0.0 (2.0±0.0)	**38.5** (2.0±0.0)
Inflammatory responses									
MMCs	1	15.4 (2.0±0.0)	15.4 (2.0±0.0)	**30.8** (2.0±0.0)	15.4 (2.0±0.0)	69.2* (2.2±0.2)	53.9* (2.3±0.3)	69.2 (2.0±0.0)	**46.2** (2.0±0.0)
Lymphocytic infiltration	2	23.1 (2.0±0.0)	15.4 (2.0±0.0)	**0.0** (0.0)	23.1 (2.0±0.0)	0.0 (0.0)	7.7 (0.0)	7.7 (2.0±0.0)	**61.5*** (2.3±0.3)
Regressive changes									
Necrosis	3	46.2 (2.3±0.3)	69.2 (2.0±0.0)	**38.5** (2.0±0.0)	**84.6** (2.0±0.0)	76.9 (2.0±0.0)	**69.2** (2.0±0.0)	84.6* (2.2±0.2)	84.6 (2.4±0.2)
Progressive changes									
HV of epithelial cells of bile ducts	2	7.7 (2.0±0.0)	0.0 (0.0)	0.0 (0.0)	7.7 (2.0±0.0)	0.0 (0.0)	7.7 (2.0±0.0)	**15.4** (3.0±1.0)	0.0 (0.0)
CPF of bile ducts	2	**23.1** (2.0±0.0)	30.8 (2.5±0.5)	30.8 (2.5±0.5)	**38.5** (2.0±0.0)	**53.8** (2.0±0.0)	46.2 (2.3±0.3)	38.5 (2.0±0.0)	38.5 (2.0±0.0)

w: lesion importance factor; MMCs: Melanomacrophage Centres; HV: Hydropic Vacuolation; CPF: Concentric Periductal Fibrosis.

Hepatocytes often appeared extensively occupied by fat vacuoles in all experimental groups (98.1%; 3.76±0.12; Figure 3E). Conversely, other hepatic lesions were always recorded at low lesion stage (Table 4). Haemorrhage, hyperaemia (Figure 3F) and hydropic vacuolation of epithelial cells of bile ducts were recorded at mild prevalence (<40%), with higher values detected in Cd exposure groups at day 7 (Table 4). Similarly, lymphocytic infiltration (Figure 3G) was observed at mild prevalence in all experimental groups (<25%), except in soles exposed to High Cd for 7 days where this lesion was moderately frequent (61.5%). Necrosis (Figure 3H) was recorded at moderate to high prevalence, with highest values detected in soles exposed to High Cd (days 3 and 7) and to Mid Cd (day 7). The prevalence of MMCs (Figure 3E) and concentric periductal fibrosis of bile ducts increased in all experimental groups from day 3 to day 7 and did not show a clear relation with Cd concentration (Table 4).

Upon microscopic examination of gonad tissue, most soles were at an early stage of gamete development. Males mostly presented immature testis (42.6%) and early spermatogenesis stage (53.2%); and although one case of early vitellogenesis (Stage II) was identified, most females (98.2%) presented primary growth oocytes (Stage I). The only histopathological lesion identified in testis was the necrosis and was recorded in soles exposed to Mid Cd for 3 d (14.3%) and High Cd for 7 d (28.6%). In females,

Table 5. Lesions prevalence (%) and means and standard errors of lesions stages for female gonad histopathological lesions identified in *S. senegalensis* collected from each experimental group at days 3 and 7. Bold values indicate significant differences between experimental groups from the same sampling time ($p<0.05$); asterisks indicate significant differences between sampling days (Pearson's Chi test, $p<0.05$).

		Day3				**Day7**			
		Control	**Low Cd**	**Mid Cd**	**High Cd**	**Control**	**Low Cd**	**Mid Cd**	**High Cd**
Gonad lesions in females	***w***	n=7	n=6	n=5	n=4	n=10	n=8	n=9	n=6
Circulatory disturbances									
Hyperaemia	1	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	**12.5** (2.0±0.0)	0.0 (0.0)	0.0 (0.0)
Inflammatory responses									
MMCs	1	0.0 (0.0)	**16.7** (2.0±0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Lymphocytic infiltration	2	0.0 (0.0)	0.0 (0.0)	20.0 (2.0±0.0)	**50.0** (2.0±0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	**16.7** (2.0±0.0)
Regressive changes									
Pyknotic oocytes	2	0.0 (0.0)	**16.7** (2.0±0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Necrosis	3	0.0 (0.0)	**33.3** (3.0±1.0)	0.0 (0.0)	0.0 (0.0)	10.0 (2.0±0.0)	25.0 (2.0±0.0)	11.1 (2.0±0.0)	**50.0** (2.0±0.0)
Atresia	3	0.0 (0.0)	0.0 (0.0)	20.0 (2.0±0.0)	**75.0** (2.0±0.0)	**10.0** (2.0±0.0)	37.5 (2.0±0.0)	22.2 (2.0±0.0)	**66.7** (2.0±0.0)
Progressive changes									
Lipids in oocytes	1	28.6 (2.0±0.0)	33.3 (2.0±0.0)	**0.0** 0.0	25.0 (2.0±0.0)	20.0 (2.0±0.0)	25.0 (2.0±0.0)	55.6 (2.4±0.4)	**100.0*** (3.0±0.4)

w: lesion importance factor.

histopathological lesions were identified at low lesion stage (≤3.0). Hyperaemia, MMCs and pyknotic oocytes were only occasionally observed (Table 5). Lymphocytic infiltration was rarely recorded, except in soles exposed to High Cd for 3 days (50%). Similarly, a mild prevalence of necrosis was recorded in most experimental groups but in High Cd exposed soles it was high at day 7 (Table 5). Highest prevalence of atresia was recorded in High Cd soles at day 3 (75.0%) and day 7 (66.7%). The prevalence of lipids in oocytes varied with Cd concentration at day 7, reaching 100% in soles exposed to High Cd (Table 5).

LS_{liver} did not differ between exposure groups. The lowest LS_{liver} was measured in the Mid Cd group after 3 d exposure (0.85±0.15) and the highest in the Mid Cd group after 7 d (1.54±0.37). Total index (I_{tot}) and liver index (I_{liver}) were significantly affected by $[Cd]_{water}$ and T; whilst the gill index (I_{gills}) and female gonad index (I_{gonad}) were only affected by $[Cd]_{water}$ (2-way ANOVA; Table 2). Thus, I_{tot} and I_{liver} increased in Cd exposed soles at day 7, with higher values at increasing Cd concentration (Figure 4A and 4C). In contrast, no significant increase was detected from day 3 to day 7 in I_{gills} Yet, higher I_{gills} values were recorded in Mid Cd and High Cd exposed soles than in Control soles at day 7 (Figure 4B). In the case of I_{gonad}, the lowest index values were recorded in Control soles and the highest values in soles exposed to High Cd (Figure 4D).

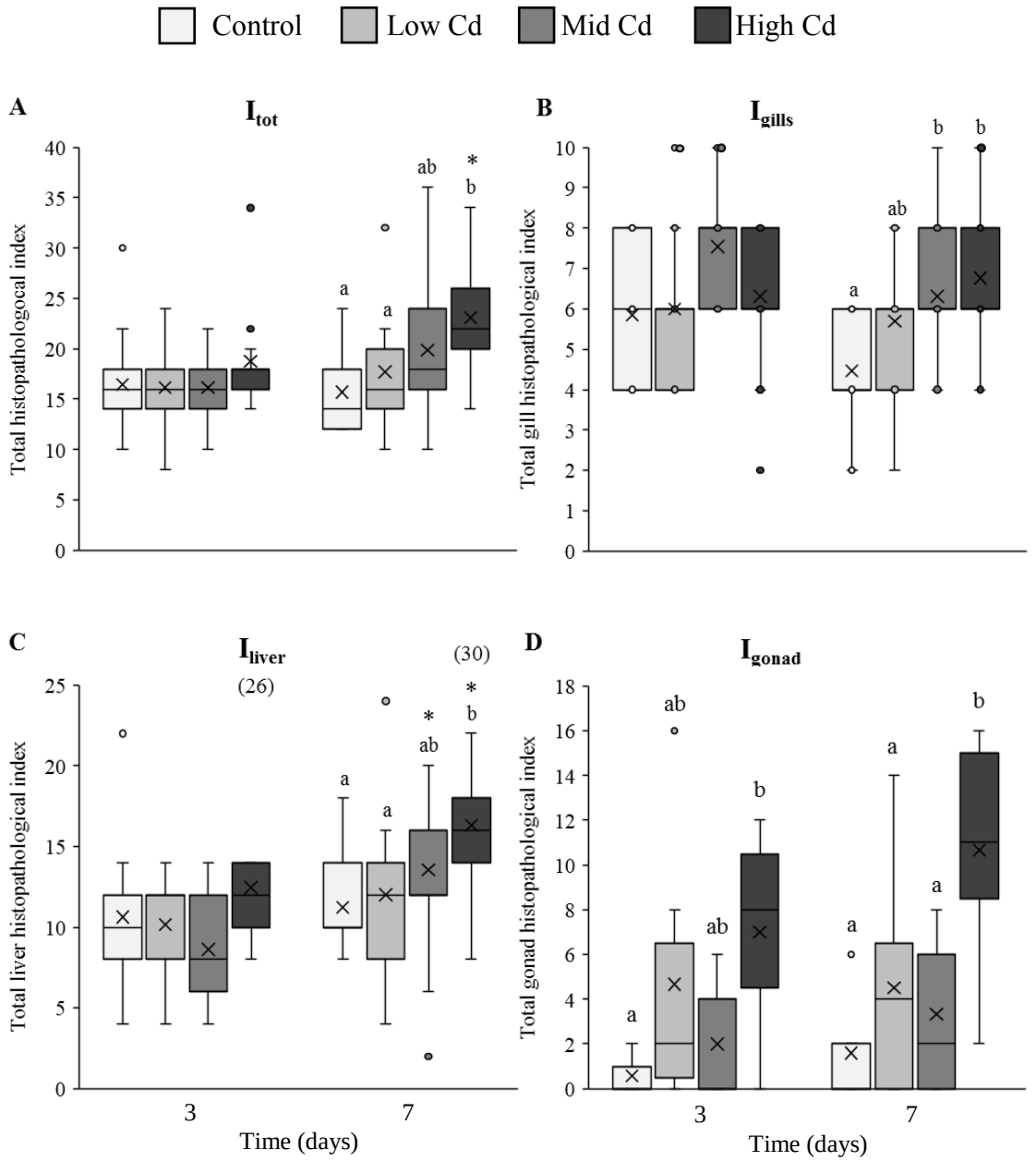
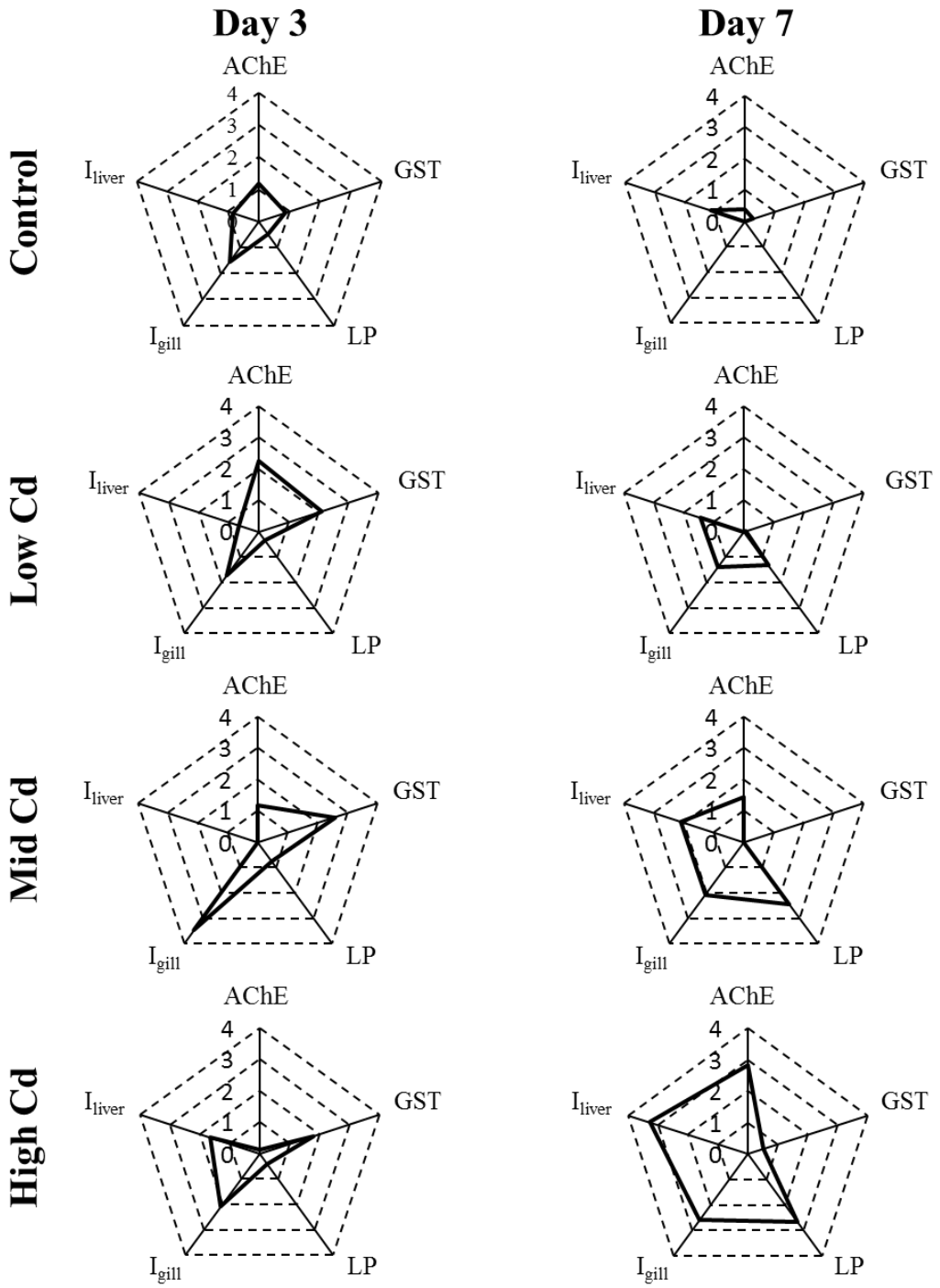


Figure 4. Total index (I_{tot}), gill index (I_{gills}), liver index (I_{liver}) and female gonad index (I_{gonad}) of *S. senegalensis* exposed to different concentrations of Cd for 3 and 7 days. Different letters indicate significant differences between experimental groups of the same sampling time; asterisks indicate significant differences between exposure times ($p<0.05$).

The IBR/n index ranged from 0.07 in Control soles at day 7 to 3.47 in soles exposed to High Cd for 7 d (Figure 5). Overall, higher IBR/n index levels were recorded in Cd exposed soles than in Control soles, in a dose dependent manner at day 7 (Control<Low Cd<Mid Cd<High Cd; Figure 5).



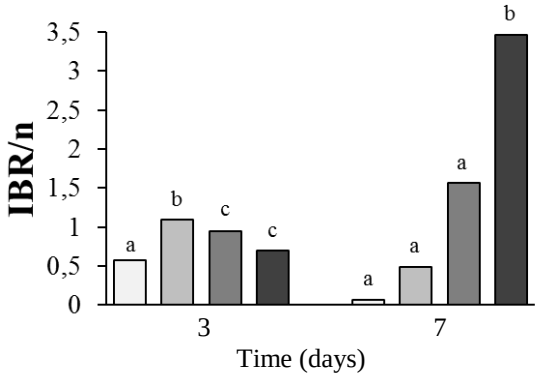


Figure 5. Radar plots constructed using five selected biological parameters (GST, AChE, LP, I_{gills} and I_{liver}) for each experimental group of juvenile *S. senegalensis* exposed to different concentrations of Cd for 3 and 7 days; and IBR/n index calculated on the basis of these radar plots.

4. Discussion

The low and mid concentrations of Cd used herein are representative of levels of this metal detected in contaminated areas where soles can be found (Eisler, 1985; Waeles et al., 2004; Costa et al., 2013). The highest concentration of Cd was used to induce rapid and acute biological effects of Cd in an attempt to identify response mechanisms to metals. The liver Cd concentration was positively correlated with waterborne Cd concentration. Moreover, upon exposure to 1000 µg Cd/l (High Cd) for 7 days it was noticeably higher than in Control soles and comparable to that reported in wild sole from a metal polluted estuary (Vicente-Martorell et al., 2009). Thus, waterborne Cd is accumulated in liver of sole juveniles, as previously reported in laboratory studies for other fish species (Hollis et al., 2001; Kim et al., 2004; Jayakumar and Paul, 2006; Kumar et al., 2008). Likewise, Cd accumulation in sole liver also occurs after dietary or intraperitoneal treatment with the metal (Kalman et al., 2010; Le Croizier et al., 2018). In contrast, no Cd was detected in muscle samples. Indeed, fish muscle usually presents limited capacity for metal accumulation (except for Hg in a few species; Jezierska and Witeska, 2006). The evidence of Cd presence in sole liver in proportion with Cd concentrations in water confirms the uptake of metal by individuals and suggests different levels of toxicity from each exposure concentration.

Neurotoxicity.

Cadmium was reported to inhibit AChE activity in brain (van der Oost et al., 2003; Davies and Vethaak, 2012; Oliva et al., 2012a). Presently, AChE activity recorded in Control soles resembles the values of the normal range elsewhere reported in sole. Thus, although they are higher than those recorded in farmed fry and adult *S. senegalensis* (Solé et al., 2012), they are absolutely comparable to those measured in wild juvenile *S. solea* from a reference site (Jebali et al., 2013). However, AChE activity was reduced to around a 30-40% in individuals exposed to 1000 µg Cd/l sw for 7 days and to a lesser extent in those exposed to 10 µg Cd/l sw. It is accepted that a 20% reduction in AChE in fish indicates exposure to neurotoxic compounds (Davies and Vethaak, 2012). This is in agreement with previous studies demonstrating the AChE inhibition in the brain of fish exposed Cd and other metals (Lavado et al., 2006; Richetti et al., 2011; Oliva et al., 2012a; Jebali et al., 2013; Ghribi et al., 2019). In comparison, exposure to environmentally relevant concentrations of 1-10 µg Cd/l does not seem to cause AChE enzyme inhibition in the short term (7 days) in the present experimental conditions; however, some signs of response on exposure to 10 µg Cd/l suggest that effects might occur at longer exposure periods.

Oxidative stress.

Cadmium accumulation in fish liver stimulates ROS production, activating antioxidant defences (e.g., CAT, GST and SOD induction; Atli et al., 2006; Souid et al., 2013). Accordingly, CAT activity was affected by exposure to Cd with values ranging 10-90 µmol/min/mg prot. In soles from the present Control group, CAT activity (≈25-30 µmol/min/mg prot) was comparable to that registered in control sole juveniles in other

experimental setups (Solé et al., 2008) and in wild sole from reference sites (Oliva et al., 2012b; Jebali et al., 2013). The transient increase of CAT activity recorded at day 3 in Cd exposed soles indicates an activation of the antioxidant defence system, particularly in High Cd exposed soles (51.1±15.9 µmol/min/mg prot). This is in agreement with observations made by Souid et al. (2013), where CAT activity was quickly induced (24 hr) in *S. aurata* exposed to waterborne Cd at a concentration similar to the highest used herein (1000 µg Cd/l). However, 7 day exposure to 1000 µg Cd/l led to a decline in CAT activity to low levels (9.6±3.7 µmol/min/mg prot), comparable to those recorded upon laboratory exposure to naturally contaminated sediments (Chapter 2) and to waterborne BaP (Chapter 4). CAT inhibition was also reported in wild juvenile soles from polluted sites (Oliva et al., 2012b). Indeed, CAT inhibition may occur in response to ROS overproduction exceeding the antioxidant protection capacity of the cell (Regoli and Principato, 1995; Roméo et al., 2000; Kalman et al., 2010). Certainly, the pattern of CAT activity in response to pollutants in fish liver may be disparate (induction or inhibition) depending on the exposure time and the concentration of chemicals (Pedrajas et al., 1996; Regoli et al., 2002). GST enzyme induction has been recorded in fish liver upon exposure to a variety of pollutants both through waterborne and sediment exposure (Regoli et al., 2002; Salamanca et al., 2008). Accordingly, a brief increase of GST activity was recorded herein in response to Cd exposure at day 3 (60-70 nmol/min/mg prot), reaching levels similar to those recorded in sole juveniles after laboratory exposure to naturally contaminated sediments (Chapter 2) and waterbone B(a)P (Chapter 4). Thus, it seems that, in all the Cd exposed soles, GST enzyme activity is at least transiently induced at day 3, indicating an activation of the antioxidant defence system (Regoli et al., 2002; Van der Oost et al., 2003). However, a longer exposure to Cd for 7 days led to a decline in GST activity (30-40 nmol/min/mg prot). Interestingly, GST inhibition was described in wild sole juveniles from highly polluted areas (Fonseca et al., 2011a). Overall, the response pattern of GST activity may be intricate depending on the chemical and on the exposure conditions (Hamed et al., 2003; Van der Oost et al., 2003; Fonseca et al., 2011a; Mani et al., 2014). For instance, in Cd exposed catfish hepatic GST activity was induced after 24 to 72h Cd exposure but inhibited after 96h exposure (Mani et al., 2014). SOD activity is enhanced upon exposure to pollutants (Pedrajas et al., 1996; Regoli et al., 2002; Wu et al., 2006; Dos Santos Carvalho et al., 2012); however it seems to be a fast transient response. For instance, SOD activity was quickly induced in *S. aurata* after 4 hr exposure to waterborne Cd (0.5 mg/l) and further on inhibited (35% decrease) within 24 hr (Souid et al., 2013). Presently hepatic SOD activity did not change with Cd exposure but was higher at day 7 (≈155 mmol/min/mg prot) than at day 3 (≈125-130 mmol/min/mg prot) in all the experimental groups. It is thus conceivable that SOD response time was shorter that 3 days and therefore, we only recorded the effects of experimentation on this enzyme activity.

Lysosomal responses.

Changes in lysosomal structure and membrane integrity are core biomarkers for biological effects assessment and they have been applied in laboratory experiments to demonstrate sub-cellular effects of Cd exposure in molluscs (Giambérini and Cajaraville

2005; Marigómez et al., 2005; Izagirre et al., 2014) and fish species (Roméo et al., 2000; Alvarado et al., 2005). Presently, LP values recorded in hepatocytes of Control soles (≈20-30 min) are similar to those previously recorded in healthy fish (Köhler et al., 1992, 1995; Broeg et al., 1999, 2002; Viarengo et al., 2007; Zorita et al., 2008). Conversely, in Cd exposed soles LP decreased from around 20 min at day 3 to 7-12min at day 7, especially in soles exposed to 1000 µg Cd/l sw. Lysosomal membrane destabilisation may occur when autophagy is enhanced to maintain cellular health status (Moore et al., 2013), once the antioxidant defence system is overloaded, as recorded herein. The reduced LP values observed after Cd exposure resemble those reported in sole and other fish species exposed to pollutants (Baršienė et al., 2006; Zorita et al., 2008; Burgeot et al., 2017; Chapters 2 and 4). Lysosomal membrane impairment may generate an outflow of hydrolases thus affecting cellular functions and potentially leading to histopathological alterations (Köhler et al., 1992, 2002; Viarengo et al., 2007). Likewise, it may be accompanied with lysosomal enlargement evidenced by changes in size and numbers of the lysosomes (Köhler et al., 1992; Cajaraville et al., 1995; Marigómez et al., 2005; Alvarado et al., 2005; Dagnino et al., 2007; Izagirre and Marigómez, 2009). However, in the present study, we have not obtained clear evidence of lysosomal enlargement. In most of the experimental groups, lysosomes observed were overall scarce and small (e.g. in comparison with control and reference individuals of other fish species; Alvarado et al., 2005; Izagirre, 2007). Similarly, although lipid accumulation in fish liver may be considered an early indicator of liver injury (Köhler et al., 2002; Köhler, 2004) the present results on intracellular accumulation of neutral lipids are not conclusive.

Histopathology.

Histopathological lesions are typically described as medium-term consequences to contaminant exposure (Bernet et al., 1999), and herein they were already recorded in gills, liver and gonads after 7 days exposure to Cd. However, overall they were recorded at low lesion stages, suggesting a low impact of Cd at tissue-level. Gill histopathology is useful for assessing early biological responses to Cd in sole (Costa et al., 2013) used to detect tissue-level effects of recent stress conditions. Gill lesions identified in the present study were reported previously in wild fish from contaminated sites (Stentiford et al., 2003; Camargo and Martinez, 2007; Oliva et al., 2013; Santos et al., 2014; Chapter 1) and after laboratory exposure to metals (Arellano, 1999; Martinez et al., 2004; Oliva et al., 2009). Hypertrophy of squamous epithelium and chloride cell hyperplasia in gills were more frequently observed in Cd exposed sole than in control, especially at day 7. Alike, haemorrhage, hyperaemia, hydropic vacuolation of epithelial cells of bile ducts, lymphocytic infiltration and necrosis were observed at higher prevalence in the liver of soles exposed to 10-1000 µg Cd/l sw for 7 days than in controls. These lesions were previously reported in fish liver upon exposure to a variety of pollutants including e.g. Cd, pesticides and B(a)P (Au, 2004; Noreña-Barroso et al., 2004; Oliveira Ribeiro et al., 2005; van Dyk et al., 2007; Chapters 2 and 4). Particularly, exposure to up to 10 µg Cd/l sw for 28 days elicited comparable histopathological lesions in the liver of younger (L: 46±7 mm) sole specimens (Costa et al., 2013). In contrast, several lesions previously described in sole in response to pollutants such as MMCs and concentric periductal

fibrosis of bile ducts (Costa et al., 2011; Zorita and Cuevas, 2014; Chapters 1, 2 and 4) were not related to the concentration of Cd in the present study. In female gonad, lymphocytic infiltration, oocyte atresia and necrosis, and lipidosis were recorded at mild lesion stages and were more frequently found in Cd exposed soles, especially upon 1000 µg Cd/l sw, than in control ones. These lesions were previously reported in fish subject to environmental stress (Blazer, 2002; Reynolds et al., 2003; Cuevas et al., 2015a; Chapters 1, 2 and 4). The use of histopathological indices integrating pathological importance and degree of severity of each lesion in different organs permitted to observe a clear general tissue-level effect of Cd. This was more remarkable in the case of the weighted histopathological indices (Bernet et al., 1999; Costa et al., 2009b). Values of I_{liver} (≈10) and I_{gonad} (0.5-2) calculated for the control soles were comparable with those measured in wild fish from non-severely contaminated areas (Cuevas et al., 2015a, 2015b). Overall, the highest indices (I_{liver}=16.3±1.3; I_{gonad}=10.7±2.2) were detected in Cd exposed soles, particularly in those exposed to 1000 µg Cd/l sw at day 7. Similarly, comparably high I_{liver} and I_{gonad} were recorded in sole juveniles exposed to contaminated sediments and to waterborne BaP exposure (Chapters 1, 2 and 4).

IBR/n index.

The use of the IBR/n index contributed to an integrated evaluation of biological responses and minimized the potential uncertainty of individual biomarkers (Marigómez et al., 2013; Baudou et al., 2019). Thus, IBR/n values were related with Cd concentration and exposure time (IBR/n_{HighCd}>IBR/n_{MidCd}>IBR/n_{LowCd}>$IBR/n_{Control}$). Alike, successive biological responses elicited by Cd were depicted by radar plot profiles: neurotoxicity and altered antioxidant enzyme activities and lysosomal system anticipated histopathological lesions. Besides, at day 3 the main contributors to IBR/n were biochemical responses related to neurotoxicity (AChE) and antioxidant defence (GST) along with gill histopathological lesions; whereas at day 7 all the biological responses in the star plots contributed to IBR/n. Likewise, a dose dependent effect was only clearly envisaged at day 7.

Concluding remarks

The assessment and integration of biological responses elicited in *Solea senegalensis* juveniles upon Cd waterborne exposure for 7 d indicated different degrees of Cd toxicity depending on concentration and time of exposure. Thus, 3-day exposure to Cd caused dose-dependent oxidative stress (CAT and GST induction/inhibition) and lysosomal membrane destabilisation, whereas neurotoxicity (AChE inhibition) and gill, liver and gonad histopathological lesions were mainly recorded at day 7. These effects were very remarkable upon exposure to a non-environmentally relevant Cd concentration (e.g. 1000 µg Cd/l sw) but also were to some extent elicited at environmentally relevant concentrations of the metal (10 µg Cd/l sw). Thus, as the effects are clearly time dependent, it is conceivable that more severe biological effects would be elicited by these low concentrations of the metal at longer exposure periods. Therefore, the present study evidences the toxicopathic effects of waterborne Cd in sole juveniles and supports the use of biomarkers and histopathology approaches as early-warning indicators of altered general health status in sole.

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Chapter IV

Biological responses and toxicopathic effects elicited in *Solea senegalensis* juveniles by waterborne exposure to benzo[a]pyrene

Congress

Briaudeau T., Zorita I., Marigómez I., Izagirre U. Lysosomal biomarkers in juvenile *Solea* spp. for early warning assessment of marine ecosystem health. 30th new European Society for Comparative Physiology and Biochemistry (ESCPB), Barcelona, España, 4-7 September 2016. Platform presentation (T. Briaudeau).

Briaudeau T., Alves Dos Santos A., Zorita I., Izagirre U., Marigómez I. A multi-organ histopathological approach in juvenile *Solea senegalensis* exposed to model contaminants in laboratory conditions: cadmium and benzo[a]pyrene. 11th Iberian and 8th Iberoamerican Congress on Environmental Contamination and Toxicology (CICTA), Madrid, Spain, 11-13 July 2018. Platform presentation (T. Briaudeau).

Briaudeau T., Alves Dos Santos A., Guerrero-Limón G., Marigómez I., Zorita I., Izagirre U. Biochemical biomarkers and histopathology in juvenile *Solea senegalensis* for early warning assessment of marine ecosystem health. SETAC Europe 28th annual meeting, Rome, Italy, 13-17 May 2018. Poster presentation.

Abstract

Organic contaminants such as polycyclic aromatic hydrocarbons (PAHs) are widely found in the aquatic environment and are considered high priority contaminants for the biota, in particular for coastal and estuarine ecosystems largely affected by anthropogenic pressure. In toxicological studies, flatfish are becoming a more prominent sentinel species for the assessment of the estuarine ecosystem health status. In the present investigation, *Solea senegalensis* juveniles were exposed to different concentrations of waterborne benzo[a]pyrene (B[a]P) using dimethyl sulfoxide (DMSO) as a carrier. Sole juveniles (n=13 per group) were retrieved after 3 and 7 d exposure. Brain, liver, gills and gonads were dissected out and processed to determine biomarkers of neurotoxicity and oxidative stress, lysosomal biomarkers and histopathology. Biological responses were consistent with waterborne B[a]P concentration and exposure time. Hepatic CAT inhibition indicated clear oxidative effects of exposure to B[a]P, even at low concentration. In comparison, exposure to low concentrations of B[a]P led to hepatic GST induction whilst higher concentrations produced enzyme inhibition, evidencing a biomarker dose-dependence. Similarly, a clear gradient in lysosomal membrane destabilisation was observed in relation with B[a]P concentration. Histopathological lesions in gills, liver and gonad were more frequent at day 7, in particular in soles exposed to higher B[a]P concentrations. In agreement, the Integrative Biological Response index (IBR/n) indicated a dose-dependent decline in health condition upon exposure to waterborne B[a]P ($IBR/n_{High\ B[a]P} > IBR/n_{Mid\ B[a]P} > IBR/n_{Low\ B[a]P} > IBR/n_{Control}$). The present investigation evidenced toxicopathic effects of waterborne B[a]P in sole juveniles and supports that biomarkers and histopathology are responsive early-warning indicators of altered general health status in sole juveniles.

Résumé

Les hydrocarbures aromatiques polycycliques (HAP) sont largement présents dans le milieu aquatique et sont des contaminants de haute priorité pour le biote marin, en particulier pour les écosystèmes côtiers et estuariens sous forte pression anthropique. Les études toxicologiques surveillant l'état de santé de ces écosystèmes se basent de plus en plus sur l'utilisation des poissons plats comme espèce sentinelle. Dans l'étude suivante, des juvéniles de *Solea senegalensis* ont été exposés à différentes concentrations de benzo[a]pyrène (B[a]P) en utilisant du diméthyl sulfoxyde (DMSO) comme vecteur. Des échantillons de cerveau, foie, branchies et gonades ont été prélevés dans chaque groupe expériemental (n=13) après 3 et 7 jours d'exposition au contaminant afin d'y analyser des biomarqueurs de neurotoxicité, de stress oxidatif et de lysosomes, ainsi que l'histopathologie des branchies, du foie et des gonades. Globalement, les réponses biologiques concordaient avec la concentration de B[a]P détectée dans l'eau et la durée d'exposition. L'inhibition enzymatique de la catalase hépatique a révélé des effets oxydatifs évidents de l'exposition au B[a]P, même à faible concentration. L'activité de la glutathion-*S*-transférase du foie variait en relation avec les concentrations de B[a]P. En effet, l'enzyme était induite par de faibles concentrations en B[a]P mais inhibée par des concentrations plus élevées. De même, la déstabilisation de la membrane lysosomale était proportionnelle à la concentration en contaminant. Les lésions histopathologiques identifiées dans les branchies, le foie et les gonades étaient plus fréquentes après 7 jours d'exposition au B[a]P, en particulier à plus fortes concentrations. L'intégration des réponses biologiques sous forme de l'index « Integrative Biological Response index » (IBR/n) a permis de détecter une diminution de l'état de santé des soles en relation avec les concentrations de B[a]P et la durée d'exposition ($IBR/n_{High\ B[a]P} > IBR/n_{Mid\ B[a]P} > IBR/n_{Low\ B[a]P} > IBR/n_{Control}$). La recherche suivante met en évidence les effets toxicopathologiques du B[a]P chez la sole juvénile et soutient l'utilisation de biomarqueurs et de l'histopathologie comme indicateurs rapides d'altérations d'état de santé de la sole face au stress environnemental.

Laburpena

Hidrokarburo polizikliko aromatikoak bezalako kutsatzaile organikoak ingurumen urtarretan aurkitu daitezke eta biotarentzako efektu handiko kutsatzaile bezala kontsideratuta daude, bereziki presio antropogenikoarengatik kaltetuta dauden itsas eta estuario ekosistematan. Arrain lauak espezie-zentinela garrantzitsuak ari dira bihurtzen ikerketa toxikologikoetan estuarioen osasuna o aztertzeko. Ikerketa honetan, *Solea senegalensis* espeziearen jubenilak dimetil sulfoxidoa (DMSO) disolbatzaile bezala erabilita uretan diluitutako benzo[a]pyreno (B[a]P) kontzentrazio desberdinetara esposatu dira. Mihi-arrain jubenilak (n=13 talde bakoitzeko) 3 eta 7 egun esposatuta egon ondoren disekzionatuak izan ziren. Garuna, gibela, zakatzak eta gonadak neurotoxikotasun eta estres oxidatibo biomarkatzaileak, biomarkatzaile lisosomikoak eta histopatologia determinatzeko prozesatu ziren. Erantzun biologikoak koherenteak ziren uretako B[a]P kontzentrazio eta esposizio denborarekin. Katalasa (CAT) hepatikoaren inhibizioak B[a]P-ren esposizioaren estres oxidatiboaren efektu argiak adierazten zituen kontzentrazio baxuetan ere. Glutation transferasa (GST) hepatikoaren aktibitateak berriz, B[a]P kontzentrazio baxuetan indukzioa eta kontzentrazio altuetan inhibizioa erakusten zuen, biomarkatzailearen dosiarekiko menpekotasuna nabarmenduz. Antzeko gradientea ikusi zen mintz lisosomikoaren desegonkortzea gora egitean B[a]P kontzentrazioarekin batera. Zakatz, gibel eta gonadetako lesio histopatologikoak ugariagoak ziren 7. egunean, bereziki B[a]P kontzentrazio handietara esposatutako mihi-arrainetan. Honekin batera, erantzun biologikoen indize integratzaileak (IBR/n), uretan disolbatutako B[a]P-aren esposizioan dosiaren menpeko osasun-baldintzaren beherakada adierazi zuen ($IBR/n_{Altua\ B[a]P} > IBR/n_{Ertaina\ B[a]P} > IBR/n_{Baxua\ B[a]P} > IBR/n_{Kontrola}$). Lan honek uretan diluitutako B[a]P-ren efektu toxipatikoak nabarmentzen ditu mihi-arrain jubeniletan eta biomarkatzaileak eta histopatologia mihi-arrainenosasun-baldintzaren alterazioen alerta-goiztiarreko indikatzaileak direla indartzen du.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are largely found in the aquatic environment and in particular in coastal and estuarine areas where the anthropogenic pressure is significant (industrial discharges, urban runoff, among other sources). Their ubiquitous presence in the aquatic environment and toxicity to the marine biota classify them as high priority contaminants for biomonitoring programmes (US EPA; Directive 2000/60/EC). Due to their hydrophobic nature, PAHs tend to concentrate in the particulate phase and to accumulate in the sediment but they can also be found dispersed in water (Hylland, 2006; Cousin and Cachot, 2014). Likewise, they can be naturally biotransformed into metabolites, potentially more toxic than the initial PAH compound (Luthe et al., 2002). Moreover, PAHs and their derivatives are known to be released from the sediment towards the water column as a result of bioturbation and biotransformation processes (Tronczynski, 1992; Zhang et al., 2000; Eggleton and Thomas, 2004; Neff et al., 2005; Chapter 2). Thus, they contribute to the waterborne exposure of marine organisms to dispersed and particulate PAHs accumulated in the sediment and their derivatives. Accordingly, bioaccumulation and toxicity of PAHs in marine organisms has been assessed by complementary diverse approaches including experimental exposure to complex mixtures of petroleum compounds suspended in water (e.g., oil water accommodated fraction WAF, chemically dispersed crude oil), to oiled sediments or to waterborne individual model compounds such as, for instance, benzo[a]pyrene (B[a]P), pyrene or anthracene (Claireaux and Davoodi, 2002; Claireaux et al., 2004; Jiménez-Tenorio et al., 2008; Salamanca et al., 2008; Solé et al., 2008; Vieira et al., 2008; Trisciani et al., 2011; Almeida et al., 2012; Ruiz et al., 2012, 2014; Cousin and Cachot, 2014; Larcher et al., 2014; Vieweg et al., 2018).

In sole, exposure to PAHs has been reported to cause altered health condition both in the field (Costa and Costa, 2008; Costa et al., 2009a, 2009b; Oliva et al., 2010; Chapter 1) and upon laboratory exposure conditions (Jiménez-Tenorio et al., 2008; Salamanca et al., 2008; Chapter 2). These laboratory studies dealt with the responses on exposure to oil WAF or oiled sediments, where the presence of chemicals in mixture may hinder data interpretation. In order to understand the impact of these cocktails of PAHs and other contaminants, these studies need to be complemented with toxicological experiments based on the waterborne exposure to individual model PAHs. B[a]P, which originates from incomplete combustion or burning of carbon-containing products, is one of the most commonly used model PAHs in ecotoxicological experimentation in marine organisms, both in molluscs (Cancio et al., 1998; Orbea et al., 2002; Marigómez and Baybay-Villacorta, 2003; Cheung et al., 2004; Marigómez et al., 2005; Speciale et al., 2018; González-Soto et al., 2019) and in fish (Hylland, 2006; Wu et al., 2006; Friesen et al., 2008; Gravato and Guilhermino, 2009; Bilbao et al., 2010 ; Costa et al., 2010a; Batel et al., 2018).

The toxicity of PAHs for marine organisms can be assessed using a battery of biochemical, cellular and tissue-level biomarkers as early warning indicators of biological

effect (UNEP/RAMOGE, 1999; Davies and Vethaak, 2012; OSPAR Commission, 2013). In fish, metabolites and reactive oxygen species (ROS) can be found accumulated in liver upon PAH exposure where they can generate oxidative stress. The response includes changes in activities of antioxidant enzymes such as glutathione-*S*-transferase (GST), catalase (CAT) and superoxide dismutase (SOD), which are considered early signals of oxidative stress in response to exposure to pollutants (Livingstone, 2001; Jee and Kang, 2005; Oliva et al., 2010). In addition, hepatic GST is also responsible for xenobiotic metabolism (Regoli and Principato, 1995; van der Oost et al., 2003; Kroon et al., 2017). Accordingly, these enzyme activities are induced in sole and other fish species on exposure to PAHs (Jiménez-Tenorio et al., 2008; Salamanca et al., 2008; Fonseca et al., 2011a; 2011b; Díaz-Garduño et al., 2018). Neurotoxicity has also been reported in fish exposed to a variety of organic compounds including PAHs and can lead to environmentally crucial effects such as alterations in behaviour (e.g. impaired swimming activity, vision or breathing) and survival (Massei et al., 2019). The inhibition of brain acetyl cholinesterase (AChE), an enzyme involved in neural transmission, is used as biomarker of neurotoxicity in sole (López-Galindo et al., 2010a, 2010b; Oliva et al., 2012a; Solé et al., 2012; Jebali et al., 2013; Siscar et al., 2013) and in other fish species (Grue et al., 1997; Heath et al., 1997; Minier et al., 2000; Davies and Vethaak, 2012; Burgeot et al., 2017). Lysosomal biomarkers are not pollutant specific but allow for the assessment of general stress responses in aquatic organisms (UNEP/RAMOGE, 1999; JAMP, 2003; ICES, 2006; Davies and Vethaak, 2012). Lysosomal enlargement, membrane destabilisation and changes in lysosomal content occur in the hepatocytes of a variety of fish species on exposure to PAHs and other pollutants (van der Oost et al., 2003; Au, 2004; Broeg et al., 2002, 2005; Alvarado et al., 2005; Einsporn et al., 2005; Baršienė et al., 2006; Bilbao et al., 2006; Zorita et al., 2008; Burgeot et al., 2017). Finally, PAHs are known to provoke histopathological lesions in fish gills and liver (Myers et al., 2003; Salamanca et al., 2008; Costa et al., 2010a; Beyer et al., 2010). In addition, gonad histology can provide further indication of environmental relevant biological effects such as reproduction impairment or endocrine disruption (Minier et al., 2000; Blazer, 2002; Reynolds et al., 2003; Bateman et al., 2004; Stentiford and Feist, 2005; WHO/UNEP, 2013; Bizarro et al., 2014; Ortiz-Zarragoitia et al., 2014; Feist et al., 2015; Ibor et al., 2016; Solé et al., 2016).

Sediment toxicity in sole seems to be related to the transfer of contaminants from the sediment to the water column, in particular for metals and PAHs (Chapter 2). A parallel investigation revealed that toxicopathic effects in sole juveniles were consistent with waterborne Cd concentration and exposure time (Chapter 3). Thus, the present investigation aimed at recognizing the toxicopathic effects elicited after waterborne expsure to B[a]P, a model individual PAH compound, in juvenile *Solea senegalensis*. Biomarkers of oxidative stress and neurotoxicity, lysosomal biomarkers and histopathology were determined after 3 and 7 d exposure and integrated into the Integrative Biological Response (IBR) index (Beliaeff and Burgeot, 2002).

2. Material and Methods

2.1 Experimental design

Solea senegalensis juveniles (24.5±1.8 cm standard length; 165.0±37.1 g total wet-wt) were exposed to different nominal concentrations of benzo[a]pyrene (B[a]P) using dimethyl sulfoxide (DMSO) as a carrier (Control; DMSO; Low B[a]P: 100 ng/l; Mid B[a]P: 1000 ng/l and High B[a]P: 100000 ng/l) for 7 d at optimal stocking density (4-6 kg/m; Schram et al., 2006). The Control group did not receive any contaminant or carrier. All other experimental groups received the same concentration of DMSO (100 µl DMSO/l sw). Each experimental group was placed in a closed-system and water changes were performed every two days to ensure optimal water conditions: pH=8, salinity=31-33 PSU, temperature=13-14ºC, dissolved O_2=6-8 mg/l and total ammonia=0 mg/l. Photoperiod throughout the experiment was set at 12:12 h light:dark. Fish were daily fed with commercial food (0.3 g per fish; BioMar Iberia S.A., Dueñas, Spain).

2.2 Chemical analysis of water samples

Water samples were collected from each experimental group, 48 hours after contaminant load. Analyses of B[a]P content in water were determined by solid-phase microextraction (SPME; Ouyang and Pawliszyn, 2006) and gas chromatography-mass spectrometry analysis (GC-MS).

2.3 Fish biometry

Individual wet-wt (W in g) and length (L in cm) and liver and gonad wet-wt (LW and GW in g, respectively) were recorded to calculate (a) $K=W\times100/L^3$; (b) HSI= LW×100/W; and (c) GSI=GW×100/W; where K is the condition factor, HSI is the hepatosomatic index, and GSI is the gonadosomatic index.

2.4 Biochemical analysis

At days 3 and 7 of exposure, liver and brain samples were dissected out, rapidly frozen and maintained at -80ºC until use. Samples were processed for biochemical analysis; they were homogenised (1:4 for liver and 1:5 for brain) in 0.1 M potassium phosphate buffer (pH 7.4) and centrifuged for 30 min at 12000 *g* at 4ºC to obtain the post-mitochondrial supernatant (PMS). Acetyl cholinesterase (AChE) activity was determined in brain PMS and glutathione-*S*-transferase (GST), catalase (CAT), superoxide dismutase (SOD) enzyme activities in liver PMS, using a BioTek Eon microplate spectrophotometer. Enzyme activities were expressed as a function of the protein concentration in the samples. Total protein content in the homogenates was measured in triplicate at 595 nm following Bradford's method adapted to microplate and using bovine serum albumin as standard (Bradford, 1976; Guilhermino et al.,1996). All enzyme assays were performed at 25ºC.

Acetylcholinesterase (AChE). AChE activity was determined according to the Ellman's colorimetric method of (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996) by measuring the formation of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) at 412 nm. The reaction medium contained 200 µl of 75 mM acetylcholine solution, 1 ml of 10 mM DTNB and 30 ml of 100 mM potassium phosphate buffer (pH 7.4). Each well contained 50 µl of samples and 250 µl of reaction medium. After 10 min of incubation, enzyme activity was recorded over 10 min. AChE activity was expressed as specific activity (nmol DTNB/min/mg protein).

Glutathione-S-transferase (GST). GST activity was determined by the Habig's method (Habig et al., 1974) adapted to microplate and using bovine serum albumin as standard (Guilhermino et al., 1996). Enzyme activity was measured following the formation of thioether by conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB). The reaction medium contained 9.9 ml of 100 mM potassium phosphate buffer (pH 7.4), 1.8 ml of 10 mM GSH solution and 300 µl of 60 mM CDNB solution. Each well contained 100 µl of samples and 200 µl of reaction medium. Enzyme activity was measured at 340 nm for 6 min and expressed as nmol/min/mg protein.

Catalase (CAT). CAT activity was determined by the method of Claiborne (1985) by measuring the rate of enzyme decomposition of hydrogen peroxide (H_2O_2) determined as absorbance decrease at 240 nm. The reaction medium (final volume of 10 ml) contained 9977 µl of 50 mM phosphate buffer (pH 7.0) and 23 µl of hydrogen peroxide (H_2O_2; 30% v/v). The reaction was started by the addition of 5 µl of samples to 295 µl of reaction medium. Absorbance decrement was measured for 3 min at 240 nm. Results were expressed as µmol H_2O_2/min/mg protein.

Superoxide dismutase (SOD). SOD activity was determined by a colorimetric method using a SIGMA kit (SOD Determination kit; ref: SIGMA 19160) to measure the superoxide anion reduction as proportional to the SOD inhibition activity. Each well contained 200 µl of WST (water soluble tetrazolium salt) working solution, 20 µl of enzyme working solution and 20 µl of sample and were left incubating at 37ºC for 20 min. Three different blanks were prepared for the assay: Blank 1 (200 µl of WST working solution, 20 µl of enzyme working solution and 20 µl of ultrapure water); Blank 2 (200 µl of WST working solution, 20 µl of dilution buffer and 20 µl of sample); and Blank3 (200 µl of WST working solution, 20 µl of dilution buffer and 20 µl of ultrapure water). Absorbance was measured at 450 nm and SOD activity (inhibition rate %) was calculated as follow:

$$\text{SOD activity (inhibition rate \%)} = \frac{(A1-A3)-(AS-A2)}{(A1-A3)} \times 100;$$

where A1 is the absorbance of Blank 1, A2 is the absorbance of Blank 2, A3 is the absorbance of Blank 3 and AS is the absorbance of the samples.

2.5 Lysosomal biomarkers

At days 3 and 7 of exposure, liver samples were dissected out, rapidly frozen and maintained at -80ºC until use. Frozen samples were processed using Tissue Array (TA) technology (Array Mold® Kit; n°20015-A) and TA blocks were cut at-27ºC using a Leica CM 3050S cryotome.

Lysosomal membrane stability (LMS). The determination of lysosomal membrane stability was based on the time of acid labilisation treatment required to produce the maximum staining intensity according to UNEP/RAMOGE (1999), after demonstration of acid phosphatase (AcP) activity in hepatocyte lysosomes. Ten serial cryotome sections (10 µm) were subject to acid labilisation in intervals of 0, 2, 4, 6, 8, 10, 15, 20, 25, 30, 40 and 50 min in 0.1 M citrate buffer (pH 4.5, containing 2.5% NaCl) in a shaking water bath at 37ºC.The demonstration of AcP activity was performed by incubation of the sections in a substrate incubation medium (naphthol AS-BI-phosphate, dimethylsulfoxide, 0.1 M citrate buffer at pH 4.5, containing 2.5% NaCl and low viscosity polypeptide, Polypep®) for 20 min at 37ºC, in a shaking bath. Rinsed sections (3% NaCl at 37ºC for 5 min) were stained at room temperature with diazonium dye Fast Violet B salt (1 mg/ml in 0.1 M phosphate buffer, pH 7.4) for 9 min. Slides were fixed in Baker's formol calcium containing 2.5% NaCl for 10 min at 4ºC, rinsed in distilled water and mounted in Kaiser´s glycerine gelatine.

The time of acid labilisation treatment required to produce the maximum staining intensity was assessed under a light microscope as the maximal accumulation of reaction product associated with lysosomes (UNEP/RAMOGE, 1999) and was denoted as the Labilisation Period (LP; in min). Four determinations were made per individual; for each area, the first maximum staining peak was considered to determine the LP value (ICES, 2015). A final LP value was calculated for each individual fish as the mean of the four LP values determined in each area.

Lysosomal Structural Changes (LSC). The determination of changes in the size and numbers of lysosomes was made according to the method described by Cajaraville et al. (1989) for mussels, further on adapted to fish liver by Alvarado et al. (2005), after histochemical demonstration of β-glucuronidase activity in fish hepatocytes. Cryotome sections (8 µm) were incubated in freshly prepared β-glucuronidase substrate incubation medium (naphthol AS-BI-β-glucuronide, 50 mM sodium bicarbonate, 0.1 M acetate buffer at pH 4.5, containing 2.5% NaCl and polyvinyl alcohol at 15%) for 20 min at 37ºC. Slides were rinsed (2.5% NaCl at 37ºC for 2 min) and transferred to a postcoupling medium (Fast Garnet, 0.1 M phosphate buffer at pH 7.4 containing 2.5% NaCl) for 10 min at room temperature, in the dark. Sections were fixed in Baker's formol calcium solution containing 2.5% NaCl for 10 min at 4 ºC, rinsed in distilled water and mounted Kaiser's glycerol gelatine.

The structure of lysosomes was assessed through a stereological procedure based on image analysis (BMS, Sevisan) according to Cajaraville et al. (1991). Five measurements

using a 100× objective lens were made per individual. The mean value of the following stereological parameters was determined for the lysosomes of each liver sample (Lowe et al., 1981): volume density ($Vv_L=V_L/V_C$), surface density ($Sv_L=S_L/V_C$), surface-to-volume ratio ($S/V_L=S_L/V_L$) and numerical density ($Nv_L=N_L/V_C$); where V=volume, S=surface, N=number, L=lysosomes and C=liver cytoplasm.

Intracellular accumulation of neutral lipids. Changes in levels of neutral lipids were determined according to Marigómez and Baybay-Villacorta (2003), after Oil Red O (ORO) staining to visualise neutral lipids (Culling, 1974). Cryotome sections (8 µm) were fixed in Baker's formol calcium containing 2.5% NaCl for 15 min at 4ºC. Air dried sections were washed in isopropanol (60%) and stained with ORO for 20 min. The staining solution (stable for 1-2 h) was freshly made from a saturated stock ORO solution (0.3% in isopropanol) and kept protected from the light. Stained sections were differentiated in 60% isopropanol, rinsed in water, counterstained with 1% Fast Green FCF for 20 min and mounted in Kaiser's glycerine.

Five measurements using a 40× objective lens were made per individual. The mean value of the volume density ($Vv_{NL}=V_{NL}/V_C$) of neutral lipids was determined; where V=volume, NL=neutral lipids and C=liver cytoplasm.

2.6 Histological processing and histopathological examination

At days 3 and 7 of exposure, gill, liver and gonad samples were dissected out (n=12 per experimental group). Gills were fixed in Bouin's solution for 24 hr at 4ºC and rinsed in formic acid (8% v/v) for 24 hr at room temperature. Liver and gonad samples were fixed in 4% neutral buffered formol for 24 hr at 4ºC. Fixed samples were dehydrated in a graded series of ethanol, cleared and embedded in paraffin (Leica ASP 300S). A minimum of two sections (5 µm) per sample were obtained using a rotary microtome (Leica RM 2125RTS) and were stained with haematoxylin-eosin (H&E; Martoja and Martoja-Pierson, 1970).

Histopathological examination. The examination of histological samples was made under a light microscope (Nikon Eclipse E200) starting with a 4× objective lens for a general description of the organs. Higher power objective lenses (10×, 20×, 40× and 100×) were used for the identification of histopathological lesions.

Hepatic samples were analysed for histopathology based on the recommendations provided by ICES (1997) and the Biological Effects Quality Assurance in Monitoring Programmes (BEQUALM, 2001). The publications by Costa et al. (2009b) on liver and gill histopathology of wild sole juveniles, Zorita and Cuevas (2014) on hepatic lesions commonly recorded in sole and Blazer (2002) on gonad histopathological lesions were used as guidelines to identify lesions in the particular case of sole. Amongst lesions identified, only persistent cases were considered for the calculation of histopathological indices and point alterations were discounted.

Histopathological indices. The semi-quantitative histopathological approach in liver was performed following two different methods.

The first approach was based on the scoring system proposed by Lang et al. (2006) for hepatic histopathology. For this purpose, hepatic lesions were classified into five categories as presented by Feist et al. (2004): (1) non-specific lesions; (2) early non-neoplastic toxicopathic lesions; (3) foci of cellular alteration (FCA); (4) benign neoplasms; and (5) malignant neoplasms. The stage (S) of each lesions recorded was determined as mild, medium and severe, depending on the size of the tissue area affected in the sections and the degree of cellular change observed. Lang's scoring system consisting of 15 lesion scores was used for the assessment of spatial and temporal variation in the lesions recorded. Lesions' scores were determined based on the lesion category and the lesion stage (S). If more than one lesion category was recorded in one specimen, the highest lesion score was used for assessment purposes. From the individual scores, mean histopathology liver lesion scores (LS_{liver}) were calculated for each sampling station and time.

The second semi-quantitative approach was based on the weighted histopathological index developed by Bernet et al. (1999). Accordingly, hepatic lesions were classified into five categories based on their reaction pattern: (1) circulatory disturbances; (2) inflammatory responses; (3) regressive changes; (4) progressive changes; and (5) tumours (neoplams). Each alteration was assigned an importance factor (w) as: (1) minimal pathological importance (the lesion is reversible after the cessation of pollutant exposure); (2) moderate pathological importance (the lesion is reversible in most cases if the exposure ends); (3) marked pathological importance (the lesion is generally irreversible and may lead to partial or total loss of organ function). The stage (a) of each lesion identified was ranked in 4 categories (0, 2, 4 and 6) according to the level of dissemination of the alteration in the organ; where 0 is absence and 6 is high degree of dissemination depending on the size of the tissue area affected in the sections and the degree of cellular change observed. Different histopathological indices were calculated using the lesion importance factor (w) and the lesion stage (a):

- organ index $I_{org.}$ was calculated for each individual and for each organ as follow:

$$I_{org} = \Sigma_{rp}\, \Sigma_{alt}\, (w_{org\ rp\ alt} \times a_{org\ rp\ alt})$$

- reaction index of an organ $I_{org\ rp}$ was calculated for each individual, each organ and each lesion category:

$$I_{org\ rp} = \Sigma_{alt}\, (w_{org\ rp\ alt} \times a_{org\ rp\ alt})$$

Gills and gonads lesions were also classified according to Bernet et al. (1999). Only I_{liver} and I_{gills} were used to calculate the total histopathological indices integrating the histopathological results from different organs:

- the total index I_{tot} was calculated for each individual, for all organs:

$$I_{tot} = \Sigma_{org}\, \Sigma_{rp}\, \Sigma_{alt}\, (w_{org\ rp\ alt} \times a_{org\ rp\ alt})$$

- the total reaction index I_{rp} was calculated for each individual and each lesion category, for all organs:

$$I_{rp}= \Sigma_{org} \Sigma_{alt} (w_{org\ rp\ alt} \times a_{org\ rp\ alt})$$

Histopathological indices. The prevalence of each histopathological alteration was determined as the percentage occurrence of an alteration within each experimental group for gills and liver and within each combination of experimental group and gender for gonads.

Characterisation of the reproductive cycle. Gonad histological sections were analysed at a light microscope for gender and gamete developmental stages determination. Male gamete developmental stages were determined according to García-López et al. (2006) and were classified in five stages as follow: Stage I (early spermatogenesis); Stage II (mid spermatogenesis); Stage III (late spermatogenesis); Stage IV (mature); Stage V (recovery). The identification of gamete developmental stages for females was mainly based on Murua and Motos (2006). Stages were classified as followed: Stage I (growth); Stage II (early vitellogenesis); Stage III (late vitellogenesis); Stage IV (maturation).

2.7 Integrative Biological Response (IBR/n) index

The IBR index (Beliaeff and Burgeot, 2002) was calculated based on the integration of biochemical (GST, CAT), histochemical (LP) biomarkers and hepatic histopathology (I_{gills}, I_{liver}) following the calculation method described by Marigómez et al. (2013). The calculation method is based on relative differences between the biomarkers in each given data set. Thus, the IBR index is computed by summing-up triangular starplot areas (multivariate graphic method) for each two neighbouring biomarkers in a given data set, according to the following procedure (Beliaeff and Burgeot, 2002; Devin et al., 2014): (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'=standardized 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 standardized value obtained for each sample to the absolute standardized value of the minimum value in the data set: $y_i = x_i' + |x_{min}'|$; (4) calculation of the Star Plot triangular areas as $A_i = (y_i \times y_{i+1} \times \sin\alpha)/2$, where y_i and y_{i+1} are the standardized values of each biomarker and its next biomarker in the star plot, respectively, and α is the angle (in radians) formed by each two consecutive axis where the biomarkers are represented in the Start Plot ($\alpha = 2\pi/n$; where n is the number of biomarkers); and (5) calculation of the IBR index which is the summing-up of all the Star Plot triangular areas ($IBR = \Sigma A_i$).Since the IBR value is directly dependent on the number of biomarkers in the data set, the obtained IBR value must be divided by the number of biomarkers used (IBR/n; Broeg and Lehtonen, 2006).

In the present work, five biomarkers were integrated for the calculation of the index as IBR/n. Parameters were selected to represent effects of waterborne Cd at different biological organisation levels where biochemical and histochemical parameters

demonstrate sub-cellular effects of contaminants and gill and liver histopathology indicate subsequent tissue-level effects.

2.8 Statistical analysis

Statistical analyses were carried out using IBM SPSS Statistics Base 22.0. Homogeneity of variance (Levene´s test) and normality of data (Shapiro's test) were tested before statistical analysis. Two-way ANOVAs were performed to analyse the effects of the B[a]P waterborne concentration ($[B[a]P]_{sw}$), the exposure time (T) and their combination ($[B[a]P]_{sw}$×T) on biomarkers and histopathology. Logarithmic transformation was applied to non-parametric variables (GST, LP, Vv_L, S/V_L and Vv_{NL}). For normal data, differences between experimental groups and throughout exposure time were tested using the parametric one-way Anova test and the T Student test, respectively. For non-normal data set, the non-parametric Kruskal-Wallis test and Mann-Whithney U test were used to analyse differences in biological data between experimental groups and throughout exposure time. The z-score test and the Pearson's Chi test were used to determine significant differences in histopathological lesions prevalence between experimental groups and throughout exposure time. Significant differences in chemical data were tested with the z-score test. Level of significance for all analyses was p=0.05.

3. Results

3.1. Chemical analysis

Overall, concentrations of B[a]P measured in water samples 48 h after contaminant load were lower than the nominal concentrations. However, the B[a]P concentration gradient was maintained. Thus, the highest waterborne concentration was measured in the High B[a]P group (17000 ng/l) and the lowest in the Low B[a]P group (40 ng/l), with the Mid B[a]P group (260 ng/l) in between. The B[a]P concentration in the DMSO and Control groups was below detection limits.

3.2. Biological responses and toxicopathic effects

In total, 130 individuals were used. Length (24.3±2.0 cm) and W (160.3±39.6 g) were constant throughout the experiment. Likewise, the condition index K (1.1±0.1), HSI (1.0±0.3) and male GSI (0.05±0.02) did not differ between exposure groups. In contrast, female GSI varied between groups at day 7, with the lowest index recorded in Mid B[a]P exposed soles and the highest in DMSO exposed soles.

3.3. Biochemical analysis

Brain AChE activity was significantly affected by $[B[a]P]_{sw}$, T and $[B[a]P]_{sw}$×T (2-way ANOVA; Table 1), varying from 110.2±2.0 nmol/min/mg prot in Mid B[a]P soles at day 3 to 66.1±3.1 nmol/min/mg prot in Low B[a]P soles at day 7 (Figure 1A). At day 3, the lowest AChE activity was recorded in Control soles and the highest in DMSO and Mid

Table 1. Summary of the 2-way ANOVAs performed to analyse the effects of $[B[a]P]_{sw}$ (d.f.: 4), time of exposure (d.f.: 1) and their combination ("$[B[a]P]_{sw}$xT", d.f.: 4) on biomarkers and histopathology (lesion stages and indices) in *S. senegalensis* exposed to different sediments for 3 and 7 days. Logarithmic transformation was applied to GST, LP, Vv_L, S/V_L and Vv_{NL} (non-parametric variable). d.f.: degrees of freedom; F: Fisher's F; *: p<0.05; **: p<0.01; ***: p<0.001.

Parameter	Residual d.f.	F($[B[a]P]_{sw}$)	F(T)	F($[B[a]P]_{sw}$xT)
AChE	50	3.134*	68.314***	9.346***
GST	51	27.579***	0.715	7.275***
CAT	52	16.157***	1.428	1.635
SOD	59	176.035***	1568.478***	23.649***
LP	105	63.576***	22.955***	1.332
Vv_L	95	0.995	38.507***	4.302**
S/V_L	95	4.287**	36.646***	5.013**
Vv_{NL}	90	3.462*	6.630*	2.427
Epithelial lifting	119	1.719	19.596***	2.025
Chloride cell hyperplasia	55	0.675	5.021*	1.218
FV of hepatocytes	126	6.412***	0.011	2.669*
LS_{liver}	129	6.130***	0.408	4.239**
I_{tot}	129	5.345**	1.370	2.477*
I_{gills}	129	4.561**	4.678*	5.322**
I_{liver}	129	3.656**	0.286	2.696*
I_{gonad}	61	6.456***	2.827	0.848

FV: Fat Vacuolation; CPF: Concentric Periductal Fibrosis. No significant effect of S, T or S×T was detected for Nv_L, gills, liver and gonad lesion stages (with the exception of epithelial lifting, chloride cell hyperplasia and FV of hepatocytes), Igills, male Igon and female Igon.

B[a]P treated soles. However, at day 7, AChE activity decreased in DMSO and B[a]P treated soles and reached levels similar to Control values.

Hepatic GST activity was affected by $[B[a]P]_{sw}$ and B[a]P×T (2-way ANOVA; Table 1). Thus, the lowest GST activity values were recorded in High B[a]P soles at day 3 and in Mid B[a]P and High B[a]P soles at day 7 (Figure 1B). In contrast, enzyme activity measured in Low B[a]P exposed soles increased at day 7. Hepatic CAT activity was significantly affected by $[B[a]P]_{sw}$ (2-way ANOVA; Table 1). Thus, the lowest CAT activity was recorded in Mid B[a]P and High B[a]P soles (Figure 1C). At day 7, soles exposed to Low B[a]P also showed lower enzyme activity than Control and DMSO soles. There were no significant differences between CAT activity values of the Control and DMSO groups. Hepatic SOD activity was affected by $[B[a]P]_{sw}$, T and B[a]P×T (2-way ANOVA; Table 1). Thus, SOD activity increased at day 7 in all the experimental groups and levels recorded in Control soles were always higher than in the other experimental groups (Figure 1D).

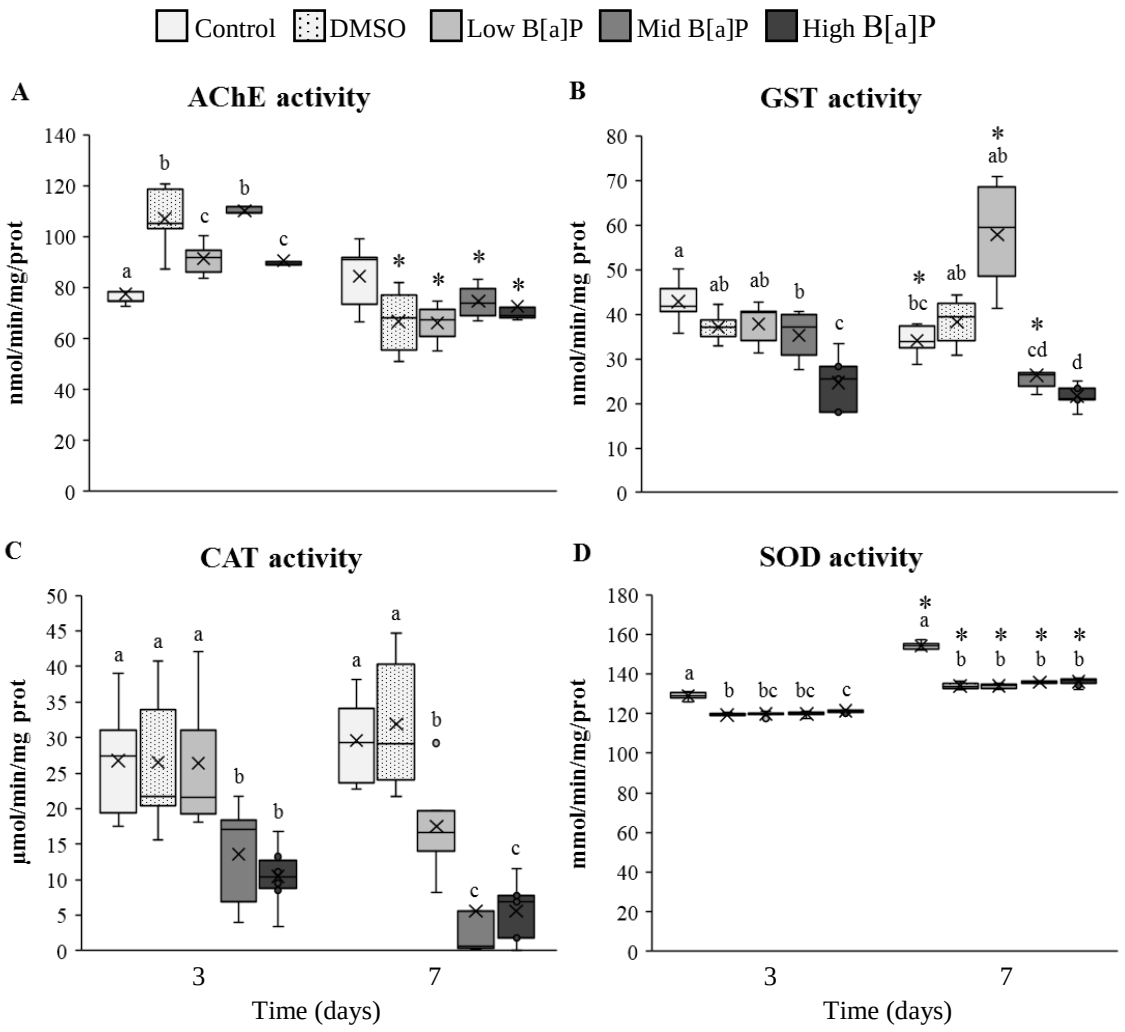

Figure 1. Brain acetylcholinesterase (A) and hepatic glutathione S-transferase (B), catalase (C) and superoxide dismutase (D) enzyme activities measured in *S. senegalensis* exposed to different concentrations of BaP for 3 and 7 days. Different letters indicate significant differences between experimental groups of the same sampling time; asterisks indicate significant differences between exposure times ($p<0.05$); AChE: acetylcholinesterase; CAT: catalase; GST: glutathione S-transferase; SOD: superoxide dismutase.

Lysosomal LP was significantly affected by $[B[a]P]_{sw}$ and T (2-way ANOVA; Table 1); ranging from 30.5±1.6 min in Control soles at day 7 to 5.7±0.4 min in High B[a]P soles at day 3 (Figure 2A). LP values from B[a]P exposed soles were significantly lower than levels recorded in the Control groups, in particular for High B[a]P exposed soles. In contrast, LP values from the DMSO group were similar to Control and Low B[a]P LP levels at day 3 and to Low B[a]P and Mid B[a]P LP levels at day 7. LP from the Control, Low B[a]P and High B[a]P groups increased at day 7. Vv_L and S/V_L were significantly affected by T and B[a]P×T and S/V_L was also affected by $[B[a]P]_{sw}$ (2-way ANOVA; Table 1). Thus, Vv_L and S/V_L were constant between experimental groups at day 3 whilst Vv_L increased and S/V_L tended to decrease in all experimental groups, except in Control soles, at day 7 (Figure 2B-C). The highest Vv_L values were recorded in DMSO soles at day 7. In contrast, Nv_L (0.00036±0.00002 $1/\mu m^3$) did not vary between experimental

groups (Figure 2D). Vv_{NL} was significantly affected by $[B[a]P]_{sw}$ and T (2-way ANOVA; Table 1). Levels of Vv_{NL} did not vary between experimental groups at day 3 and were maintained constant in Control and High B[a]P soles (Figure 2E). In contrast, higher Vv_{NL} values were recorded in the other experimental groups at day 7, in particular in DMSO treated soles.

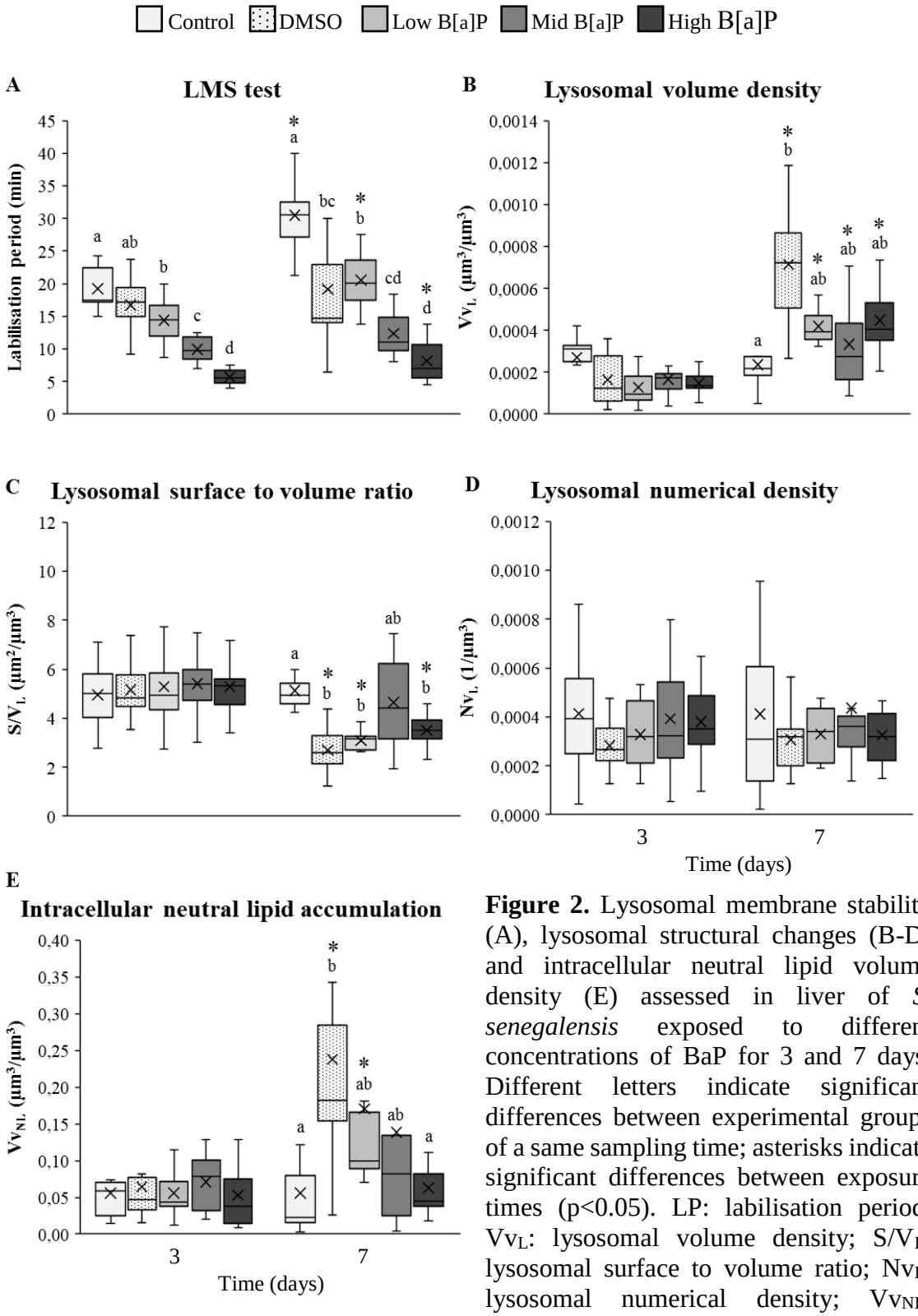

Figure 2. Lysosomal membrane stability (A), lysosomal structural changes (B-D) and intracellular neutral lipid volume density (E) assessed in liver of *S. senegalensis* exposed to different concentrations of BaP for 3 and 7 days. Different letters indicate significant differences between experimental groups of a same sampling time; asterisks indicate significant differences between exposure times ($p<0.05$). LP: labilisation period; Vv_L: lysosomal volume density; S/V_L: lysosomal surface to volume ratio; Nv_L: lysosomal numerical density; Vv_{NL}: volume density of neutral lipids.

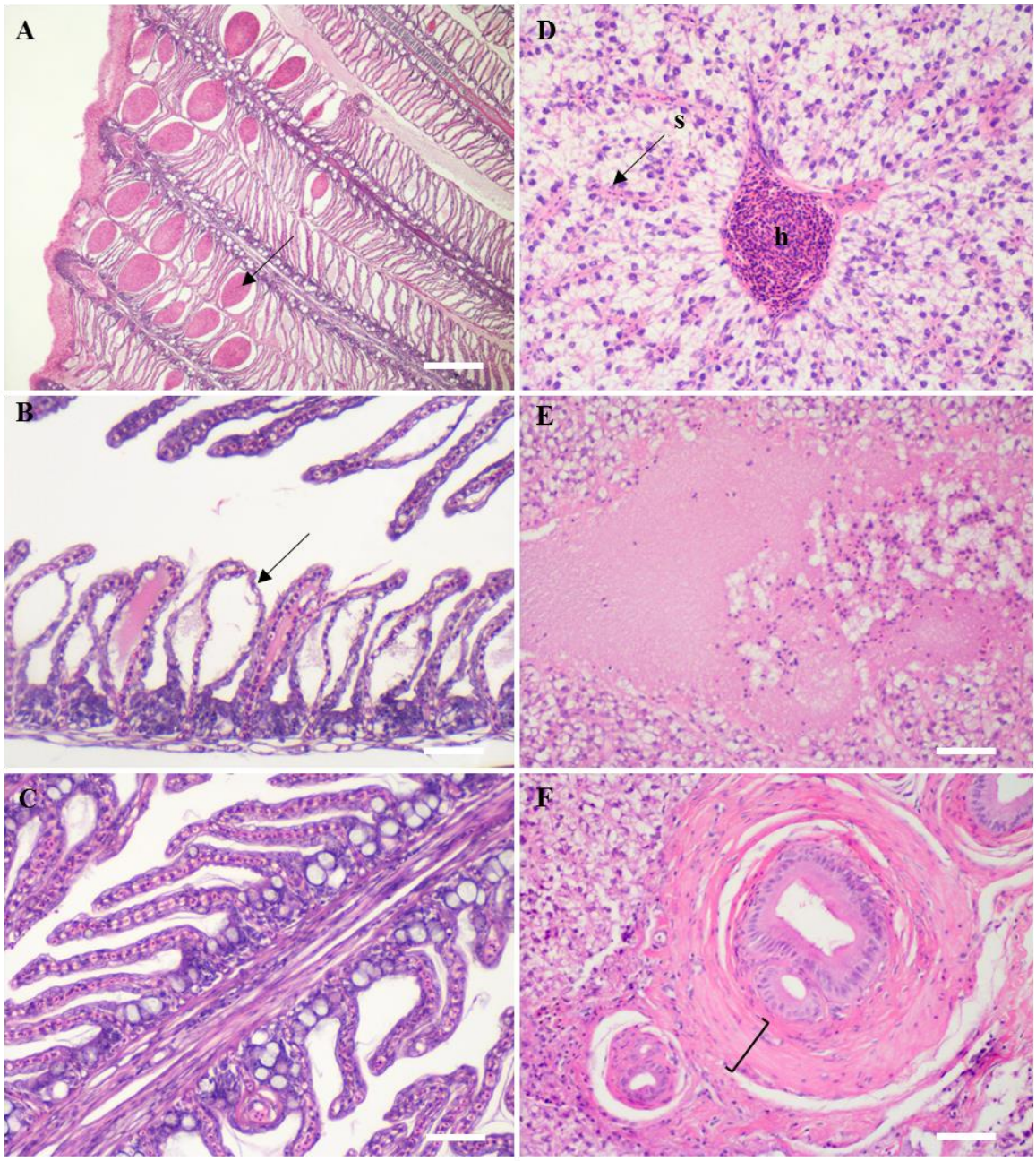

Figure 3. Histological sections (5 µm) of *S. senegalensis* exposed to different concentrations of BaP for 3 and 7 days, stained with haematoxylin and eosin. (A) Gill tissue showing capillary aneurysm (arrow); (B) gill epithelial lifting (arrow); (C) chloride cell hyperplasia; (D) hepatic hyperaemia with accumulation of erythrocytes in blood vessels; h: hyperaemia; s: sinusoid; (E) severe case of hepatic necrosis; (F) Concentric Periductal Fibrosis. Black scale bar: 50 µm; white scale bar: 100 µm.

Practically all the examined soles presented lamellar capillary aneurysm and epithelial lifting in the gills (Figure 3A-B). Hypertrophy of squamous epithelium was detected at mild to high prevalence and chloride cell hyperplasia (Figure 4C) at low to mild prevalence (Table 2).The lowest prevalence of hypertrophy of squamous epithelium was recorded in the Control group. Lesion stages of epithelial lifting and chloride cell hyperplasia were significantly affected by T (2-way ANOVA; Table 1).

Table 2. Lesions prevalence (%) and means and standard errors of lesions stages (n=13; lesion intensity when detected) for gill histopathological lesions identified in *S. senegalensis* collected from each experimental group at days 3 and 7. Bold values indicate significant differences between experimental groups from the same sampling time ($p<0.05$); asterisks indicate significant differences between sampling days ($p<0.05$).

		Day3					Day7				
Gill lesions	***w***	**Control**	**DMSO**	**Low BaP**	**Mid BaP**	**High BaP**	**Control**	**DMSO**	**Low BaP**	**Mid BaP**	**High BaP**
Circulatory disturbances											
Lamellar capillary aneurysm	1	100.0 (2.5±0.2)	100.0 (2.1±0.2)	100.0 (2.2±0.2)	**76.9** (2.0±0.0)	100.0 (2.2±0.2)	92.3 (2.2±0.2)	**76.9** (2.2±0.2)	84.6 (2.0±0.0)	100.0 (2.2±0.2)	100.0 (2.3±0.2)
Regressive changes											
Epithelial lifting	1	92.3 (2.3±0.2)	**75.0** (2.2±0.2)	92.3 (2.3±0.3)	100.0 (2.5±0.2)	100.0 (2.3±0.2)	**76.9** (2.6±0.3)	100.0 (3.1±0.4)	92.3 (3.0±0.3)	100.0 (2.9±0.3)	100.0 (4.0±0.2)*
Progressive changes											
Hypertrophy of squamous epithelium	1	**53.8** (2.3±0.3)	76.9 (2.0±0.0)	76.9 (2.2±0.2)	61.5 (2.0±0.0)	**84.6** (2.0±0.0)	**23.1** (2.0±0.0)	84.6 (2.0±0.0)	53.8 (2.0±0.0)	84.6 (2.5±0.3)	76.9 (2.0±0.0)
Chloride cell hyperplasia	2	46.2 (2.0±0.0)	**69.2** (2.0±0.0)	46.2 (2.0±0.0)	**38.5** (2.0±0.0)	53.8 (2.3±0.3)	23.1 (2.7±0.7)	38.5 (2.4±0.4)	**69.2** (2.2±0.2)	30.8 (3.0±0.6)	15.4* (2.0±0.0)

w: lesion importance factor.

Table 3. Lesions prevalence (%) and means and standard errors of lesions stages (n=13; lesion intensity when detected) for liver histopathological lesions identified in *S. senegalensis* collected from each experimental group at days 3 and 7. Bold values indicate significant differences between lesion prevalence of experimental groups from a same sampling time ($p<0.05$); different letters indicate significant differences between lesions stages of experimental groups from a same sampling time ($p<0.05$); asterisks indicate significant differences between sampling days ($p<0.05$).

		Day3					Day7				
Hepatic lesions	***w***	**Control**	**DMSO**	**Low BaP**	**Mid BaP**	**High BaP**	**Control**	**DMSO**	**Low BaP**	**Mid BaP**	**High BaP**
Circulatory disturbances											
Haemorrhage	1	7.7 (2.0±0.0)	0.0 (0.0)	7.7 (2.0±0.0)	0.0 (0.0)	**15.4** (2.0±0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	**7.7** (2.0±0.0)	**7.7** (2.0±0.0)
Hyperaemia	1	23.1 (2.0±0.0)	15.4 (2.0±0.0)	23.1 (2.0±0.0)	**7.7** (2.0±0.0)	15.4 (2.0±0.0)	7.7 (2.0±0.0)	0.0 (0.0)	0.0 (0.0)	15.4 (3.0±1.0)	**23.1** (2.0±0.0)
Inflammatory responses											
MMCs	1	**15.4** (2.0±0.0)	61.5 (2.5±0.5)	53.8 (2.9±0.4)	61.5 (2.3±0.3)	61.5 (2.3±0.3)	69.2* (2.2±0.2)	**61.5** (2.0±0.0)	**76.9** (2.4±0.4)	**61.5** (2.3±0.3)	**76.9** (2.4±0.4)
Lymphocytic infiltration	2	23.1 (2.0±0.0)	**15.4** (2.0±0.0)	**30.8** (2.0±0.0)	**30.8** (2.0±0.0)	**15.4** (2.0±0.0)	0.0 (0.0)	0.0 (0.0)	15.4 (2.0±0.0)	**53.8** (2.0±0.0)	7.7 (2.0±0.0)
Regressive changes											
Necrosis	3	**46.2** (2.3±0.3)	69.2 (2.2±0.2)	61.5 (2.3±0.3)	76.9 (2.0±0.0)	**84.6** (2.2±0.2)	76.9 (2.0±0.0)	**61.5** (2.0±0.0)	76.9 (2.2±0.2)	76.9 (2.0±0.0)	**61.5** (2.0±0.0)
Progressive changes											
FV of hepatocytes	1	100.0 (4.3±0.4)	100.0 (4.6±0.3)	100.0 (4.9±0.4)	**92.3** (4.7±0.4)	100.0 (5.5±0.2)	**92.3** (3.3±0.4)^{a}	100.0 (4.9±0.4)^{bc}	**92.3** (4.8±0.3)^{b}	100.0 (5.8±0.2)^{c}*	100.0 (5.2±0.4)^{bc}
HV of epithelial cells of bile ducts	2	7.7 (2.0±0.0)	7.7 (2.0±0.0)	**0.0** (0.0)	7.7 (2.0±0.0)	**15.4** (2.0±0.0)	0.0 (0.0)	0.0 (0.0)	**38.5*** (2.4±0.4)	0.0 (0.0)	0.0 (0.0)
CPF of bile ducts	2	23.1 (2.0±0.0)	30.8 (2.5±0.5)	30.8 (2.0±0.0)	16.7 (2.7±0.7)	**69.2** (2.0±0.0)	53.8 (2.0±0.0)	38.5 (2.0±0.0)	**69.2*** (2.0±0.0)	53.8 (2.0±0.0)	38.5 (2.0±0.0)

w: lesion importance factor; MMCs: Melanomacrophage Centres; FV: Fat Vacuolation; HV: Hydropic Vacuolation; CPF: Concentric Periductal Fibrosis.

Table 4. Lesions prevalence (%) and means and standard errors of lesions stages (n=13; lesion intensity when detected) for female gonad histopathological lesions identified in *S. senegalensis* collected from each experimental group at days 3 and 7. Bold values indicate significant differences between experimental groups from the same sampling time ($p<0.05$); asterisks indicate significant differences between sampling days ($p<0.05$).

		Day3					**Day7**				
		Control	**DMSO**	**Low BaP**	**Mid BaP**	**High BaP**	**Control**	**DMSO**	**Low BaP**	**Mid BaP**	**High BaP**
Gonad lesions in females	***w***	n=7	n=6	n=8	n=7	n=5	n=10	n=4	n=6	n=4	n=5
Circulatory disturbances											
Hyperaemia	1	0.0	**16.7**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		(0.0)	(2.0±0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)
Inflammatory responses											
Lymphocytic infiltration	2	0.0	16.7	0.0	**42.9**	20.0	0.0	25.0	0.0	**50.0**	0.0
		(0.0)	(2.0±0.0)	(0.0)	(2.0±0.0)	(2.0±0.0)	(0.0)	(2.0±0.0)	(0.0)	(2.0±0.0)	(0.0)
Regressive changes											
Necrosis	3	**0.0**	50.0	25.0	**57.1**	20.0	**10.0**	75.0	16.7	75.0	60.0
		(0.0)	(2.0±0.0)	(2.0±0.0)	(2.5±0.5)	(2.0±0.0)	(2.0±0.0)	(2.0±0.0)	(2.0±0.0)	(2.0±0.0)	(2.0±0.0)
Atresia	3	0.0	**16.7**	0.0	0.0	0.0	10.0	0.0	0.0	25.0	**60.0***
		(0.0)	(2.0±0.0)	(0.0)	(0.0)	(0.0)	(2.0±0.0)	(0.0)	(0.0)	(2.0±0.0)	(2.0±0.0)
Progressive changes											
Lipids in oocytes	1	28.6	**16.7**	37.5	42.9	40.0	20.0	**0.0**	50.0	50.0	40.0
		(2.0±0.0)	(2.0±0.0)	(2.0±0.0)	(2.0±0.0)	(2.0±0.0)	(2.0±0.0)	(0.0)	(2.0±0.0)	(3.0±1.0)	(2.0±0.0)

w: lesion importance factor; n: sample size for female gonad histopathology.

Higher lesion stages of epithelial lifting were detected at day 7, in particular in High B[a]P exposed soles (Table 2). No tumours were detected in gills.

Often, liver samples appeared largely vacuolated showing important lipid accumulation in hepatocytes in all experimental groups. Haemorrhage, hyperaemia (Figure 3D) and lymphocytic infiltration were rarely observed in Control and DMSO soles but they were occasionally identified in B[a]P exposed soles (Table 3). Cases of melanomacrophage centre accumulation (MMCs), necrosis (Figure 3E) and concentric periductal fibrosis (Figure 3F) were detected at moderate prevalence in most groups and in particular in High B[a]P exposed soles (Table 3). The lesion stage of fat vacuolation in hepatocytes was moderate-to-high in all the soles examined; however, it was significantly affected by $[B[a]P]_{sw}$ and $[B[a]P]_{sw}$×T (2-way ANOVA; Table 1). Thus, the highest lesion stages were recorded at day 7, in particular in soles exposed to Mid B[a]P and High B[a]P (Table 3). On the other hand, all the other hepatic lesions were recorded at mild lesion stage (Table 3) and did not change during experimentation (2-way ANOVA; Table 1).

Upon microscopic examination of gonad tissue, most soles were shown to be at an early stage of gamete development. Males mostly presented immature testis (46.3%) and early spermatogenesis stage (50.7%); and although one case of early vitellogenesis (Stage II) was identified, most females (98.4%) presented primary growth oocytes (Stage I). No histopathological lesions were identified in testis of Control soles. Moreover, the only lesions recorded in testis of DMSO and B[a]P exposed soles were scarce with only few cases of granulomatous tissue (<20.0%) and necrosis (<35.0%).

In females, lymphocytic infiltration, necrosis, atresia and pre-vitellogenic oocyte lipidosis were rarely detected in Control soles and were at mild to moderate prevalence in the other experimental groups. Highest prevalence levels for these lesions were measured in soles exposed to Mid B[a]P or High B[a]P (Table 4). Lesions stages were low (<4.0) and did not vary between experimental groups (2-way ANOVA; Table 1, Table 4).

LS_{liver} was significantly affected by $[B[a]P]_{sw}$ and B[a]P×T (2-way ANOVA; Table 1); ranging from 1.62±0.18 (Control group, at day 7) to 3.08±0.26 (Low B[a]P group, at day 7). Thus, LS_{liver} from Control soles decreased at day 7 and was lower than in the other experimental groups (Figure 4).

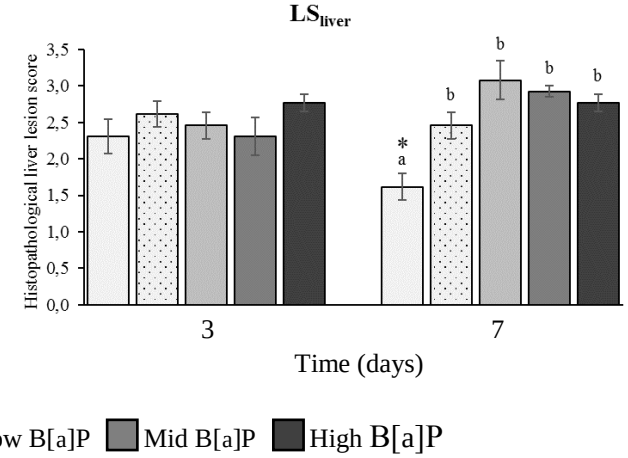

Figure 4. Histopathology liver lesion scores (LS_{liver}) of *S. senegalensis* exposed to different concentrations of BaP for 3 and 7 days. Different letters indicate significant differences between experimental groups of the same sampling time; asterisks indicate significant differences between exposure times ($p<0.05$).

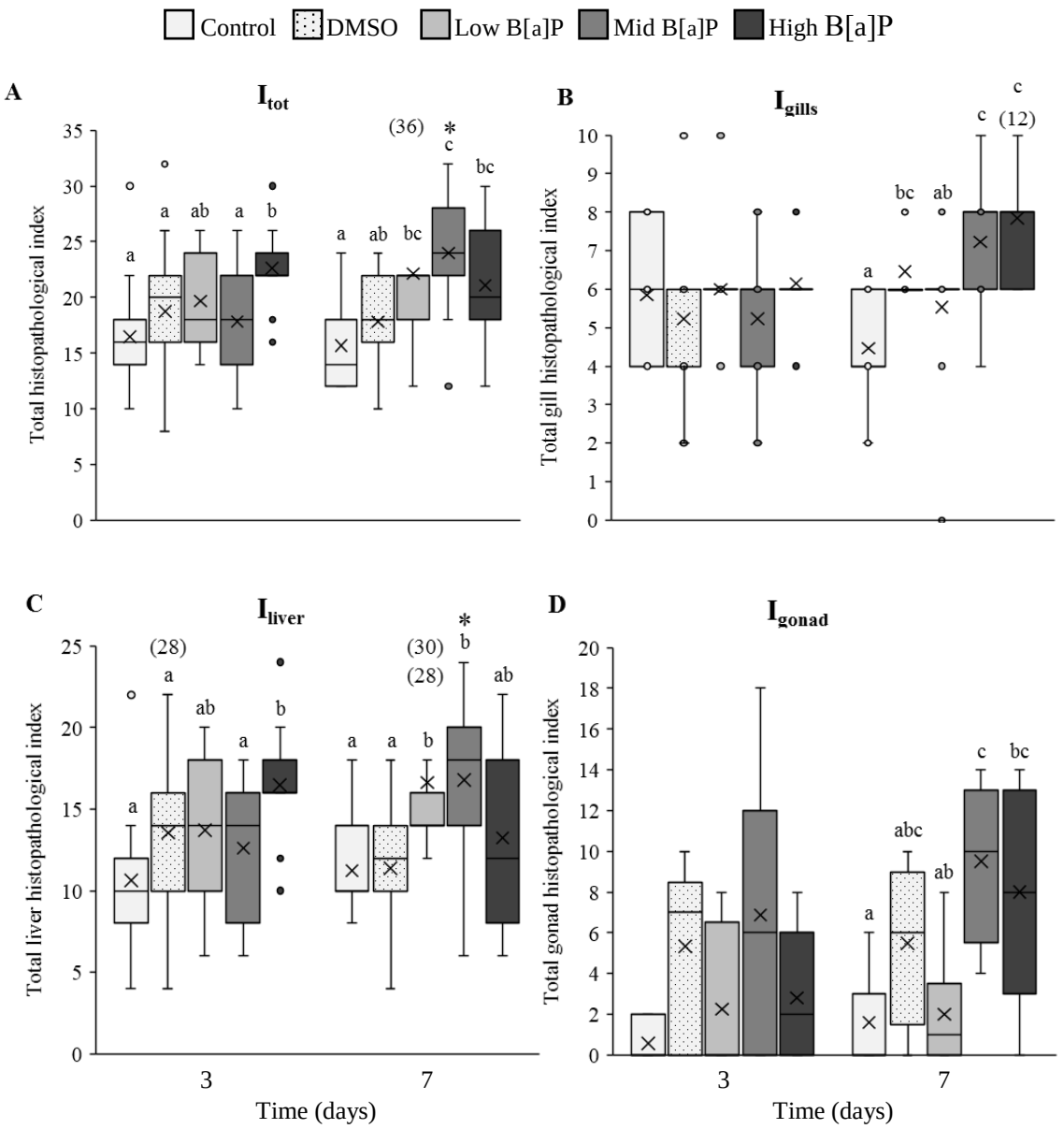

Figure 5. Total index (I_{tot}), gill index (I_{gills}), liver index (I_{liver}) and female gonad index (I_{gonad}) of *S. senegalensis* exposed to different concentrations of BaP for 3 and 7 days. Different letters indicate significant differences between experimental groups of the same sampling time; asterisks indicate significant differences between exposure times ($p<0.05$).

I_{tot}, I_{gills} and I_{liver} were significantly affected by $[B[a]P]_{sw}$ and B[a]P×T; whereas I_{gills} also varied with T (2-way ANOVA; Table 1). I_{tot}, I_{gills} and I_{liver} from the Control and DMSO groups were similar whilst higher values were recorded in B[a]P exposed soles, in particular at day 7 (Figure 5A-C). Similarly, female I_{gonad} was significantly affected by B[a]P (2-way ANOVA; Table 1). Although female I_{gonad} did not differ between experimental groups at day 3, highest values were recorded in Mid B[a]P and High B[a]P groups at day 7 (Figure 5D). The highest male I_{gonad} was recorded in individuals from the DMSO group, at day 7 (4.67±1.94) but significant differences were not detected between experimental groups (2-way ANOVA; Table 1).

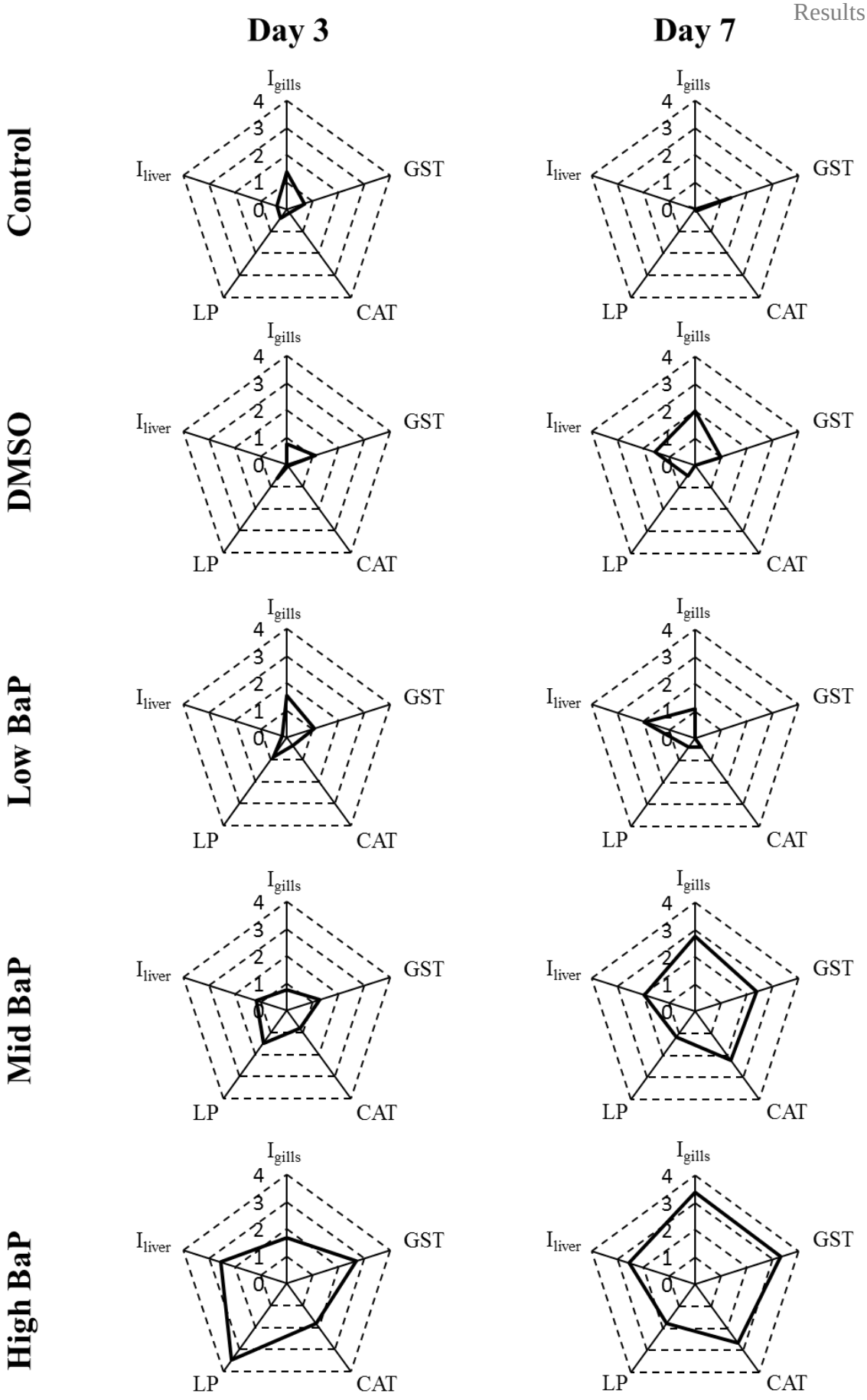

Figure 6A. Radar plots constructed using five selected biological parameters (GST, CAT, LP, I_{liver} and I_{gills}) for each experimental group of juvenile *S. senegalensis* exposed to different concentrations of BaP for 3 and 7 days.

The IBR/n index ranged from 0.01 in Control soles at day 7 to 3.63 in soles exposed to High B[a]P for 7 d (Figure 6A-B). Higher IBR/n index levels were recorded in High B[a]P soles at days 3 and 7 and in Mid B[a]P soles at day 7.

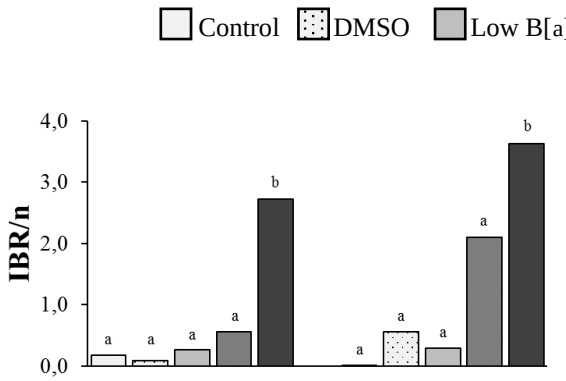

Figure 6B. IBR/n index calculated on the basis of the radar plots constructed using five selected biological parameters (GST, CAT, LP, I_{liver} and I_{gills}) for each experimental group of juvenile *S. senegalensis* exposed to different concentrations of BaP for 3 and 7 days.

4. Discussion

The application of individual model PAH compounds like B[a]P for ecotoxicological studies implies the need of a carrier to counteract the hydrophobicity of the contaminant. DMSO has been previously applied as an effective solvent vehicle for organic compounds that is considered to have a low toxicity for fish species (Willford, 1967). The OECD recommends a maximum DMSO concentration of 1.1 mg/l to avoid biological effects of the carrier in fish. Hutchinson et al. (2006) provided a review of previous studies testing the toxicity of low concentrations of DMSO in aquatic animals. Among them, Pawlowski et al. (2004a, 2004b) demonstrated that DMSO at 0.01% (v/v) may affect egg production in fathead minnow but did not alter liver and gonad histology. Similarly, after long-term exposure (100 days) to 0.01-2.0% (v/v) DMSO did not generate histopathological lesions in gills and kidney in Coho salmon (Benville et al., 1968). Thus, the DMSO concentration (100 µl DMSO/l sw) used herein was not expected to elicit confounding biological responses in sole juveniles. Accordingly, only minor differences were occasionally found between soles of Control (seawater) and DMSO experimental groups. Yet, exposure to DMSO for 7 days seemed to have some influence on SOD enzyme activity and lysosomal biomarkers. Nevertheless, although the toxicity of the carrier used herein cannot be fully neglected it is worth noting that the responses to B[a]P exposure were clearly demonstrable in comparison with the Control and DMSO groups.

The B[a]P concentrations used in the present study were selected to ensure that biological effects would be exerted in sole juveniles, as it could be expected after preceding reports on other fish species (Vieira et al., 2008; Gravato and Guilhermino, 2009). Chemical analysis of water samples permitted to confirm that the B[a]P concentrations in experimental tanks were congruent with the gradient of nominal concentrations originally selected. Yet, the measured concentrations were noticeably lower than the nominal ones; this might be due to the low solubility of B[a]P in seawater or to the loss of waterborne B[a]P (samples were taken 48 hr after dosing) either by relatively fast evaporation during the running of the experiment at 18ºC and with aeration in open tanks or by binding to

solid matter. Indeed, heavy PAHs (<3 rings) such as B[a]P occur at low concentration in soluble forms in seawater due to their low seawater solubility and to their strong binding to particulate matters and solid materials (Qingling et al., 2006). In agreement, waterborne PAH concentration in experimental setups is known to be reduced as a result of their adsorption to particulate matter or to the basin surface, as well as due to their photochemical degradation, biotransformation and uptake by fish (Budzinski et al., 2004). Presently, the consequences of these confounding factors in the mid-term were somehow buffered because seawater and B[a]P were renewed every second day, thus contributing to maintain for 7 days the differences in the B[a]P exposure level between the experimental groups even though the measured concentrations of the PAH were lower than the nominal ones.

Neurotoxicity.

AChE enzyme activity recorded in soles from the seawater Control group was similar to values reported in farmed individuals (Solé et al., 2008). However, a decrease in AChE activity was recorded at day 7 on exposure to DMSO and to different concentrations of B[a]P. AChE enzyme inhibition was reported previously in fish *Pomatoschistus microps* exposed to B[a]P (Vieira et al., 2008) and in *S. senegalensis* exposed to the biocide sodium hypochlorite (López-Galindo et al., 2010a). Overall, exposure to PAHs such as B[a]P is known to inhibit AChE activity in fish brain (Baršienė et al., 2006); however, to our knowledge DMSO was not expected to cause neurotoxic effects at the used concentrations (Yen et al., 2011). Nevertheless, behavioural toxicity of the carrier was reported in early life stage of other fish species exposed to higher DMSO concentration (Chen et al., 2011) and therefore a certain neurotoxicity of this compound cannot be fully disregarded for sole juveniles under the present experimental conditions. In any case, the present findings indicate that more research is needed to properly use DMSO or other alternative carriers in experimental setups in which the toxicity of waterborne PAHs (or that of other hydrophobic organic chemicals) is investigated.

Biotransformation of organic contaminants.

GST activity values recorded herein for Control and DMSO exposed soles resemble those reported for farmed individuals (Solé et al., 2008) and for wild sole from reference sites (Oliva et al., 2012b; Jebali et al., 2013). On this basis, GST was seemingly induced upon exposure to 100 ng B[a]P/l, which suggests that Phase II detoxification processes were activated. Indeed, GST is a Phase II enzyme known to contribute to PAH biotransformation (van der Oost et al., 2003). Alike, GST induction was described in other fish species after exposure to waterborne B[a]P (Vieira et al., 2008; Gravato and Guilhermino, 2009) and in sole juveniles after exposure to oiled sediments (Salamanca et al., 2008). In contrast, under the present experimental conditions exposure to waterborne B[a]P concentrations in the range of 1000-100000 ng B[a]P/l caused GST inhibition. Almeida et al. (2012) also reported that GST enzyme activity decreased in juvenile seabass exposed to waterborne pyrene. In agreement with the present observations, Gravato and Guilhermino (2009) also reported that GST enzyme activity was either induced or inhibited in seabass depending on the contaminant concentration: GST activity

was induced after exposure to a nominal concentration of 4000 ng B[a]P/l in seawater and inhibited by higher concentrations (8000-16000 ng/l). Thus, the decrease in GST activity observed herein may imply that the capacity of sole juveniles to detoxify B[a]P and its metabolites through the pathway of glutathione conjugation is limited when waterborne concentrations of this individual PAH compound exceed a nominal concentration of 1000 ng B[a]P/l. Interestingly, this concentration is comparable to the range of B[a]P concentration reported in highly contaminated estuarine waters, which in addition occur along in combination with other chemicals (Maskaoui et al., 2002).

Oxidative stress.
Exposure to contaminants induces CAT activity in fish liver indicating the activation of antioxidant defences (Jee and Kang, 2005; López-Galindo et al., 2010a, 2010b; Oliva et al., 2012b; Chapter 3). However, CAT activity can result also inhibited when the overproduction of ROS exceeds the antioxidant capacity of the cell (Roméo et al., 2000; Kalman et al., 2010; Oliva et al., 2012b). Presently, exposure to a nominal concentration of B[a]P in the range of 1000-100000 ng B[a]P/l clearly inhibited CAT activity at day 3, whereas the inhibition was more attenuated and recorded only after 7 days upon exposure to the nominal concentration of 100 ng B[a]P/l. Therefore, the degree oxidative stress seems to vary depending on the B[a]P concentration, with early and more marked ROS excess in sole juveniles exposed to a waterbone nominal concentration of B[a]P higher than 100 ng B[a]P/l (measured 48 hr-$[B[a]P]_{sw}$ = 40 ng B[a]P/l). Likewise, as a parallel evidence of the antioxidant capacity overload at those exposure concentrations, lysosomal responses revealed signs of membrane disruption and potential pathogenesis (see below). A comparable profile of pollutant concentration dependent induction/inhibition profile was observed in sole juveniles exposed to waterborne Cd (Chapter 3). Regarding SOD activity, obtained results are not conclusive as changes in this enzyme activity could not be related to B[a]P exposure; quite the contrary, SOD activity was lower in DMSO and B[a]P exposed soles that in control ones and higher at day 7 than at day 3 in all the experimental groups. Inconsistent results have been reported in other studies and seem to be related to the complexity and timing of the antioxidant defence network. Thus, for instance, SOD induction was reported in fish upon exposure to waterborne B[a]P (2000-20000 ng B[a]P/l) for 6 days, SOD was inhibited after 12 days exposure to 20000 ng B[a]P/l (Wu et al., 2006).

Lysosomal responses.
Preceding laboratory experiments revealed that individual PAH compounds like B[a]P can provoke lysosomal enlargement and membrane destabilisation in marine organisms (Marigómez and Baybay-Villacorta, 2003; Marigómez et al., 2005; Zorita et al., 2008). Present LP values recorded in Control and DMSO groups (≈15-30 min) are similar those previously recorded in healthy fish (Köhler et al., 1992; Köhler and Pluta, 1995; Broeg et al., 1999, 2002; Viarengo et al., 2007; Zorita et al., 2008). Meanwhile, reduced LP values recorded in B[a]P exposed soles range from 14.42±0.98 (100 ng B[a]P/l) to 5.68±0.39 min (100000 ng B[a]P/l), with a clear concentration-dependent profile. These values are similar to those reported in wild *Platichthys flesus* affected by an oil spill event (Baršienė et al., 2006) and in wild *Limanda limanda* collected from the polluted Seine estuary (Burgeot et al., 2017). Altered lysosomal membrane stability is commonly accompanied with lysosomal structure changes evidenced by changes in size and numbers of the

lysosomes (Köhler et al., 1992; Cajaraville et al., 1995; Marigómez et al., 2005; Alvarado et al., 2005; Dagnino et al., 2007; Izagirre and Marigómez, 2009). Yet, in the present study lysosomal enlargement (high Vv_L and low S/V_L) was evident only in DMSO and B[a]P exposed soles at day 7, especially in the former. A comparable profile was observed regarding intracellular accumulation of neutral lipids. It seems therefore that the carrier exerted some effects on juvenile soles, thus rendering present results on changes in lysosomal structure and content not fully conclusive regarding B[a]P effects.

Histopathology.

Gill lesions identified in the present study were similar to those previously described in wild fish from contaminated sites (Stentiford et al., 2003; Camargo and Martinez, 2007; Oliva et al., 2013; Santos et al., 2014; Chapter 1) and from laboratory experiments using organic compounds (Jímenez-Tenorio et al., 2008; Martins et al., 2016). Overall, higher lesion prevalence were recorded in B[a]P exposed soles than in Control or DMSO groups at day 7. Similarly, higher lesion stage of epithelial lifting was recorded at day 7, in particular in soles exposed to 100000 ng B[a]P/l. Haemorrhage, hyperaemia, increased MMCs, lymphocytic infiltration, hydropic vacuolation of epithelial cells of bile ducts and concentric periductal fibrosis, and necrosis were observed at higher prevalence in B[a]P exposed soles than in Control or DMSO groups. These lesions were previously described in sole, both after laboratory exposure to pollutants and in field studies (Costa et al., 2011, 2013; Oliva et al., 2013; Zorita and Cuevas, 2014; Chapters 1, 2 and 3). In testis, only few cases of granulomatous tissue and necrosis were recorded, with no clear relation with B[a]P exposure; in agreement, histopathological lesions in testis were also only circumstantial in sole juveniles exposed to Cd (Chapter 3). In contrast, lymphocytic infiltration, necrosis, atresia and lipids in oocytes were identified at mild to moderate prevalence in the ovaries of B[a]P exposed soles, especially upon exposure to 1000-100000 ng B[a]P/l. These lesions were previously reported in fish subject to environmental stress (Blazer, 2002; Reynolds et al., 2003; Cuevas et al., 2015a, 2015b; Chapters 1, 2 and 3). Lesions were integrated into weighted histopathological indices (Bernet et al., 1999; Van Dyk et al., 2007; Costa et al., 2009a) to identify potential cause-effect relationship between exposure to B[a]P and disease condition in sole juveniles. Thus, higher total index (I_{tot}), gill index (I_{gills}) and liver index (I_{liver}) were recorded in soles exposed to B[a]P than in the Control and DMSO groups, in particular at day 7. In agreement, previous works reported increased histopathological indices after exposure to pollutants (Bernet et al., 2004; Van Dyk et al., 2007; Jímenez-Tenorio et al., 2008; Costa et al., 2009a; Chapter 1). Moreover, the highest I_{liver} (≈16) and I_{gonad} (≈10) values recorded upon exposure to 1000-100000 ng B[a]P/l are not dissimilar from the values reported in sole juveniles exposed to contaminated sediments and to waterborne Cd (Chapters 1, 2 and 3).

IBR/n index.

IBR/n values clearly showed that the biological responses and toxicopathic effects elicited by waterborne B[a]P on sole juveniles were dose and time dependent ($IBR/n_{HighB[a]P} > IBR/n_{MidB[a]P} > IBR/n_{LowB[a]P} > IBR/n_{DMSO} > IBR/n_{Control}$; especially at day 7).

The successive biological responses elicited by B[a]P were depicted by radar plot profiles: altered antioxidant enzyme activities and lysosomal system anticipated histopathological lesions. At day 3, the main contributors to IBR/n were biochemical responses related to antioxidant defence (GST and CAT) along with altered lysosomal system and to a lesser extent, liver histopathology. Meanwhile, all the selected biological responses contributed to IBR/n at day 7. Whilst the response profile in sole juveniles exposed to 100 ng B[a]P/l was comparable to the one depicted in the DMSO group, exposure to nominal concentrations of B[a]P in the range of 1000-100000 ng B[a]P/l provoked inhibition of GST and CAT enzyme activities, severe lysosomal membrane destabilisation and outstanding hitopathological lesions in liver and, most remarkably, in gills.

Concluding remarks

The assessment and integration of biological responses elicited in *Solea senegalensis* juveniles upon B[a]P exposure for 7 d indicated different degrees of B[a]P toxicity depending on the waterborne B[a]P concentration and the exposure time. Whilst a 3-day exposure to a nominal concentration in the range of 1000-100000 ng B[a]P/l caused oxidative stress (CAT inhibition; GST induction/inhibition) and lysosomal membrane destabilisation, a 7-day exposure also caused gill, liver and gonad histopathological lesions. Upon exposure to 100 ng B[a]P/l, the effects were not distinguishable from those elicited by the experimental carrier DMSO. However, they were elicited both upon exposure to a non-environmentally relevant B[a]P concentration (e.g. 100000 ng B[a]P/l) and also upon exposure to an environmentally relevant concentration (1000 ng B[a]P/l). Therefore, it seems that environmentally relevant waterborne concentrations of B[a]P would suffice to cause toxicopathic effects on sole juveniles in relatively short exposure times. Moreover, if B[a]P alone is a potential toxicopathic pollutant, its co-occurrence in the water column with other PAHs and chemicals derived from polluted sediments (as described in Chapters 1 and 2) would be expected to represent a veiled but real hazard that should entail future research efforts.

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IV. General Discussion, Conclusions and Thesis

General Discussion

The suitability of flatfish as sentinel species to monitor the health status of coastal and estuarine ecosystems has been demonstrated for various species of Northern Europe (Köhler et al., 1992; Myers et al., 1994; Vethaak et al., 1996; Myers et al., 2003; Stehr et al., 2003; Stentiford et al., 2003; Lang et al., 2006). However, these species are not so abundant along the Iberian Peninsula and the Bay of Biscay, which is an inconvenient for their use in biomonitoring programmes of this region. Instead, *Solea* spp. is commonly found along the N-E Atlantic coasts. In particular, juveniles concentrate in estuarine and coastal areas for the first 2-3 yr of life. Thus, sole juveniles and adults have been introduced in previous field studies to assess the health status of coastal and estuarine ecosystems (Jebali et al., 2013; Oliva et al., 2010, 2012a, 2012b, 2013, 2014; Gonçalves et al., 2013, 2014; Siscar et al., 2013, 2015; Cuevas et al., 2015a, 2015b; Vieira et al., 2018). Most of these studies assessed biological effects of environmental contamination by comparing levels of responses (mainly based on biochemical and histopathological approaches) in soles collected from different sampling sites and changes in the health status of sole were rarely surveyed throughout time (Oliva et al., 2010; Cuevas et al., 2015a, 2015b; Vieira et al., 2018). The application of monitoring campaigns for several years is essential to identify biological effects of environmental contamination and to survey the potential recovery of the ecosystem over time.

The possible implementation of a long-term monitoring programme using sole juveniles was demonstrated in Chapter 1 for the Bilbao estuary. Enough individuals ($n \geq 30$) of similar size were obtained annually for 7 years. The Bilbao estuary was used as a case study for the assessment of the health status of an estuarine ecosystem experiencing recovery after intense contamination, based on sediment chemistry and multi-organ sole histopathology. Although industrial decline and improvement of wastewater-treatment of the area allowed for the recovery of water and sediment quality, contaminants can still be found trapped in the sediment (Wolanski and Richmond, 2008). Indeed, chemical analysis of the sediment suggested an overall decrease in contaminants concentration throughout the year with episodes of chronical contamination by metals, PCBs and PAHs (Montero et al., 2013; Borja et al., 2015). This fluctuating toxicity of the sediment was reflected on changes in the health status of sole juveniles, assessed by liver, gills and gonad histopathology. Liver histopathology revealed alterations of mild severity and suggested an overall improvement in environmental conditions of the estuary, in agreement with sediment chemistry. Nevertheless, gills which are considered as an organ indicator of early biological responses, elicited histopathological alterations in the recent years of campaigns suggesting new environmental threat (e.g. input of new contaminant, remobilization of older contaminants trapped in the sediment). The 7-yr monitoring programme of the Bilbao estuary using sole juveniles confirms the possibility of surveying the health status of the ecosystem, based on sediment chemistry and sole juveniles' histopathology. However, the identification of a clear temporal trend in data from sediment contamination or sole juveniles' health was intricate. Indeed, the influence of other parameters such as water contamination and environmental factors (e.g.

temperature, salinity, pH) may also generate changes in sediment contamination and ecosystem health status. In the field, the implementation of long-term monitoring programmes is essential to overcome the complexity of the ecosystem and identify general trends in environmental quality/health. Moreover, ecotoxicology assays in laboratory conditions can complement field studies to elucidate the association between water and sediment contamination and changes in the health status of juvenile soles.

Chapter 2 aimed at relating contaminants mixture in natural sediments with biological responses elicited in sole juveniles upon laboratory exposure. Chemical analysis demonstrated that each sediment used for the study reacted differently in experimental conditions affecting their toxicity. The release of contaminants from the sediment to the water column varied depending on the presence of chemicals as a mixture and the concentration, speciation and mobility of each chemical. This inconsistency in sediment toxicity supports the need for biological approaches to assess the overall toxicopathic effects of sediment contamination. Biological responses in sole juveniles were assessed after 7 and 28 d of exposure, at different levels of biological complexity based on biochemical (CAT, SOD, GST, AChE) and lysosomal biomarkers (lysosomal enlargement and membrane stability, intracellular neutral lipid accumulation), and multi-organ histopathological approaches (liver, gills and gonad). Overall, oxidative stress (CAT inhibition, GST induction), alterations in lysosomal responses (lysosomal enlargement, membrane destabilisation and changes in lysosomal content) and multi-organ histopathology were elicited by all the sediments. Differences in sediment toxicity were suggested for particular biomarkers and were more clearly defined upon the integration of biomarkers assessed at different levels of biological complexity. Exposure to relatively low sediment contamination induced the antioxidant defence system (CAT and GST induction) with no severe biological effects recorded at higher level of biological complexity. In contrast, higher sediment contamination incited early oxidative stress (CAT inhibition), which was reflected onto the lysosomal system (altered lysosomal structure, membrane destabilisation and content). These early biological effects were maintained in time and passed at higher levels of biological complexity with tissue-level responses detected in different organs, in particular in the liver. The present study confirms the suitability of sediment toxicity assays to elucidate the association between the presence of a mixture of contaminants and toxicopathic effects recorded in sole juveniles based on chemical analysis, biochemical, cellular and tissue-level biomarkers, including histopathology.

As demonstrated in Chapters 1 and 2, alterations in the health status of sole can be complex to interpret in the presence of a mixture of contaminants as encountered in sediments and/or in the water column. Thus, toxicity assays using single contaminants (e.g. model metals and PAHs) at different concentrations allow to identify levels of biological responses and are essential to support biological effects of contamination recorded in the field. Chapter 3 aimed to assess toxicopathic effects of different concentrations of Cd in sole juveniles after 3 and 7 d of exposure. Overall, biological responses assessed in Cd exposed soles were consistent with the contaminant

concentrations, as established for the experiment and confirmed by chemical analysis, and exposure time. A 3-day exposure to different concentrations of Cd caused oxidative stress (CAT and GST induction) with no severe biological effects at higher level of biological complexity. At day 7 however, Cd toxicopathic effects included dose-dependent oxidative stress (CAT and GST inhibition), neurotoxicity (AChE inhibition), and lysosomal membrane destabilisation. The lysosomal membrane destabilisation is a core biomarker recognised by UNEP and recommended for marine monitoring programmes for several marine organisms (OSPAR Commission, 2010; Davies and Vethaak, 2012; HELCOM, 2012; UNEP/MAP, 2014); the present experiment demonstrates its suitability for sole juveniles to assess cellular effects of exposure to different concentrations of Cd, including environmental levels. In comparison, changes in lysosomal structure recorded in sole hepatocytes upon Cd exposure were more ambiguous. Alterations recorded at cellular level were reflected at higher level of biological complexity with histopathological alterations detected in different organs (liver, gills and gonad). The present toxicity assay elucidates the association between concentrations of Cd and exposure time with subsequent toxicopathic effects assessed in sole juveniles, based on the integration of a battery of biomarkers assessed at different levels of biological complexity.

Similarly, Chapter 4 aimed to identify levels of biological responses in sole juveniles exposed to different concentrations of a model PAH (B[a]P) for 3 and 7 days. Overall, toxicopathic effects of B[a]P recorded in soles were consistent with contaminant concentration and exposure time. A 3-day exposure to different concentrations of B[a]P caused oxidative stress (changes in CAT and GST activities) and lysosomal membrane destabilisation. Thus, as observed upon exposure to Cd, the core lysosomal biomarker is suitable to assess cellular effects of exposure to B[a]P in sole juveniles, even at environmental concentrations. Early biological effects of B[a]P exposure recorded at day 3 were maintained at day 7 and passed to higher biological level of complexity with histopathological alterations detected in different organs (liver, gills and gonad). Unlike in the case of Cd exposure, B[a]P neurotoxicity could not be clearly identified here as the contaminant carrier (DMSO, 100 µl DMSO/l sw) seemed to slightly interfere with the activity of this enzyme. Nevertheless, the integration of biological responses assessed at different levels of biological complexity and based on different biological endpoints clearly illustrated the time and dose-dependent toxicity of B[a]P in sole juveniles. The present toxicity assay elucidates the association between concentrations of B[a]P and exposure time with levels of biological responses recorded in sole juveniles based on a integrative biomarker approach.

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Conclusions

1. The 7-yr monitoring programme of the estuary of Bilbao permitted to survey the health status of an ecosystem experiencing recovery after intense contamination, based on sediment chemistry and sole juveniles' histopathology. Although the estuary is involved in a recovery process, levels of contaminants detected in the sediment fluctuated over the years and indicated that the ecosystem was still chronically impacted by metals, PCBs and PAHs.

2. Toxicological adverse effects of the Bilbao estuary pollution were reflected on the health status of sole juveniles based on histopathological approaches. Liver histopathological lesions were of mild severity and suggested an improvement in environmental conditions of the estuary, in agreement with sediment chemistry. In contrast, gills considered as an organ indicator of early biological responses, elicited histopathological alterations in the recent years of campaigns suggesting new environmental threat (e.g. input of new contaminant, remobilization of older contaminants trapped in the sediment).

3. The identification of temporal trends in Bilbao estuary sediment contamination and in histopathological alterations in sole juveniles is intricate. The assessment of the ecosystem health status of the estuary demands for the implementation of long-term monitoring programmes, the application of ecotoxicology assays in laboratory conditions and the development of a battery of biomarkers for a better interpretation of contaminant toxicopathic effects elicited in sole juveniles.

4. The complexity of sediment contamination relies on the presence of chemicals as a mixture; the concentration, speciation and mobility of each chemical influences the overall toxicity profile of the milieu. Sediment granulometry, OM content, redox potential and chemical analysis are essential data to assess and survey sediment contamination.

5. Laboratory exposure to contaminated sediments caused a clear decline in the health status of sole juveniles. Although sediment toxicity proved to vary under experimental conditions, the overall biological responses elicited in sole were representative of sediment toxicity confirmed by chemical analysis. Toxicity was recorded for all the sediments through the determination of biological effects as oxidative stress (CAT inhibition, GST induction), alterations in lysosomal responses (lysosomal enlargement, membrane destabilisation and changes in lysosomal content) and multi-organ histopathology. Differences in sediment toxicity were suggested for individual biomarkers especially observed in CAT enzyme activity and in lysosomal membrane stability at day 7 and liver histopathology at day 28. This association between sediment contamination and biological effects was clearly defined at day 28, particularly upon the integration of the battery of biomarkers assessed at different levels of biological complexity.

6. The suitability of whole-sediment toxicity assays to elucidate the association between the presence of contaminants in sediments and the emergence of toxicopathic effects in sole juveniles based on chemical analysis, biomarkers and histopathological approaches is confirmed.

7. Impairment in the health status of sole juveniles exposed to Cd were concomitant to contaminant concentration and exposure time. A 3-day exposure to Cd caused a dose-dependent oxidative stress (CAT and GST induction) with no evident effects at higher biological levels of complexity. At day 7, biological effects of Cd were maintained and passed to higher biological levels of complexity with dose-dependent oxidative stress (CAT and GST inhibition), neurotoxicity (AChE inhibition), altered lysosomal system (lysosomal membrane destabilisation) and multi-organ histopathology. The integration of biological responses assessed based on different biological endpoints clearly illustrates the time and dose dependent toxicity of Cd in sole juveniles.

8. Impairment in the health status of sole juveniles exposed to B[a]P were related to contaminant concentration and exposure time. A 3-day exposure to B[a]P caused a dose-dependent oxidative stress (CAT and GST inhibition) and lysosomal membrane destabilisation. At day 7, toxicopathic effects of B[a]P exposure were maintained and spread to higher biological levels of complexity as evidenced by multi-organ histopathology. The integration of biological responses assessed at different levels of biological complexity and based on different biological endpoints clearly illustrates the time and dose dependent toxicity of B[a]P in sole juveniles.

9. The suitability of toxicity assays using waterborne model contaminants (Cd and B[a]P) at different concentrations (below and above environmentally effective concentrations) to identify toxicopathic effects of environmental stressors based on a battery of biomarkers in sole juveniles is confirmed.

Thesis

Solea spp. is responsive to environmentally realistic concentrations of waterborne pollutants, both dissolved and released from sediments, which can be quantified upon de application of a "biomarkers+histopathology" toolbox; therefore, sole is suitable as sentinel species for the assessment of the biological effects of pollution in OSPAR Region IV biomonitoring programmes in the context of EU Marine Strategy Framework Directive.

V. Appendix

Appendix

1. Biochemical protocols

1.1. Samples processing

0.1 M potassium phosphate buffer (pH 7.4)
Dissolve 17.42 g of potassium phosphate dibasic (K_2HPO_4) in 1000 ml of distilled water. Dissolve 0.265 g of monosodium phosphate (KH_2PO_4) in 1000 ml of distilled water. Mix both solutions and set the pH at 7.4 using NaOH and HCl (at 0.1M, 1M and 5M).

- Dilute each samples in 0.1 M potassium phosphate buffer (pH 7.4) in a 1:4 ratio for liver samples and 1:5 ratio for brain samples.
- Homogenise the samples (Precellys homogenizer at 6500 rpm for 30 sec at 4ºC).
- Centrifuge the samples at 12 000 *g* for 30 min and extract the supernatant.
- Dilute the samples using 0.1 M potassium phosphate buffer (pH 7.4) in 1:5 for the analysis of Catalase and Superoxide Dismutase, in 1:20 for the analysis of Acetylcholinesterase in brain, in 1:40 for the analysis of Glutathione-*S*-transferase.

1.2. Protein quantification (Bradford)

- The dye reagent used for this protocol should be brought to room temperature before use.
- Pipet 5 µl of samples in each well, preparing three replicates per sample including the blank samples and the pre-made standard samples. Two sets of blank samples should be prepared with distilled water and dye reagent.
- Add 250 µl of dye reagent.
- Incubate at room temperature for 5 min.
- Read the absorbance at 595 nm.

1.3. Acetylcholinesterase (AChE)

0.1 M potassium phosphate buffer (pH 7.4)
Dissolve 17.42 g of potassium phosphate dibasic (K_2HPO_4) in 1000 ml of distilled water. Dissolve 0.265 g of monosodium phosphate (KH_2PO_4) in 1000 ml of distilled water. Mix both solutions and set the pH at 7.4 using NaOH and HCl (at 0.1M, 1M and 5M).

0.075 M acetylcholine solution
Dissolve 0.10835 g of acetylthiocholine ($C_7H_{16}INOS$, M=289.7 g/mol; SIGMA A5751) in 5 ml of ultra-pure water. The solution should be kept protected from the light and stored in the fridge (for maximum five days).

10mM DTNB solution
Dissolve 0.0198 g of DTNB ($C_{14}H_8N_2O_8S_2$; M=196.3 g/mol; SIGMA D8130) and 0.0075 g of $NaHCO_3$ (M=84.01 g/mol; MERCK 6329) in 5 ml of 0.1 M potassium phosphate buffer (pH 7.4). The solution should be stored in the fridge (for maximum five days).

Reaction solution
Mix 30 ml of 0.1 M potassium phosphate buffer (pH 7.4) with 0.2 ml of 0.075 M acetylcholine solution and 1 ml of 10mM DTNB solution. The solution should be freshly prepared at the time of use.

- Prepare the reaction medium.
- Pipet 50 µl of samples including blank samples (0.1 M potassium phosphate buffer, pH 7.4) in triplicate.
- Add 250 µl of reaction medium.
- Incubate for 10 min.
- Read the absorbance at 412 nm for 10 min.

1.4. Glutathione-*S*-transferase (GST)

0.1 M potassium phosphate buffer (pH 7.4)
Dissolve 17.42 g of potassium phosphate dibasic (K_2HPO_4) in 1000 ml of distilled water. Dissolve 0.265 g of monosodium phosphate (KH_2PO_4) in 1000 ml of distilled water. Mix both solutions and set the pH at 7.4 using NaOH and HCl (at 0.1M, 1M and 5M).
10 mM GSH solution
Dissolve 30.73 mg of GSH in 10 ml of 0.1 M potassium phosphate buffer (pH 7.4). The solution should be freshly prepared and kept in ice.

60mM CDNB solution
Dissolve 60.78 mg of CDNB in 5 ml ethanol. This solution should be freshly prepared and kept protected from the light.

Reaction medium
Mix 9900 µl of 0.1 M potassium phosphate buffer (pH 7.4) with 1800 µl of 10 mM GSH solution and 300 µl of 60mM CDNB solution.

- Pipet 100 µl of samples including blank samples (0.1 M potassium phosphate buffer, pH 7.4) in triplicate.

- Add 200 µl of reaction medium to each well.

- Read the absorbance at 340 nm every 40 sec for 6 min.

1.5. Catalase (CAT)

Reaction medium
Mix 9977 µl of 0.05 M potassium phosphate buffer (pH 7.0) with 23 µl of H_2O_2 (30% v/v).

- Prepare the standard solutions according to the following table.
- Pipet 5 µl of samples.
- Add 295 µl of reaction medium
- Read the absorbance at 240 nm every 40 sec for 3 min.

Standard Curve	*0.05 M potassium phosphate buffer*	Reaction medium
1	0	1000
2	400	600
3	600	400
4	800	200
5	900	100
6	950	50
7	975	25
8	1000	0

1.6. Superoxide Dismutase (SOD)

This analysis was made using the SOD Determination kit (SIGMA 19160).

WST working solution
Dilute 1 ml of WST Solution with 19 ml of Buffer Solution.

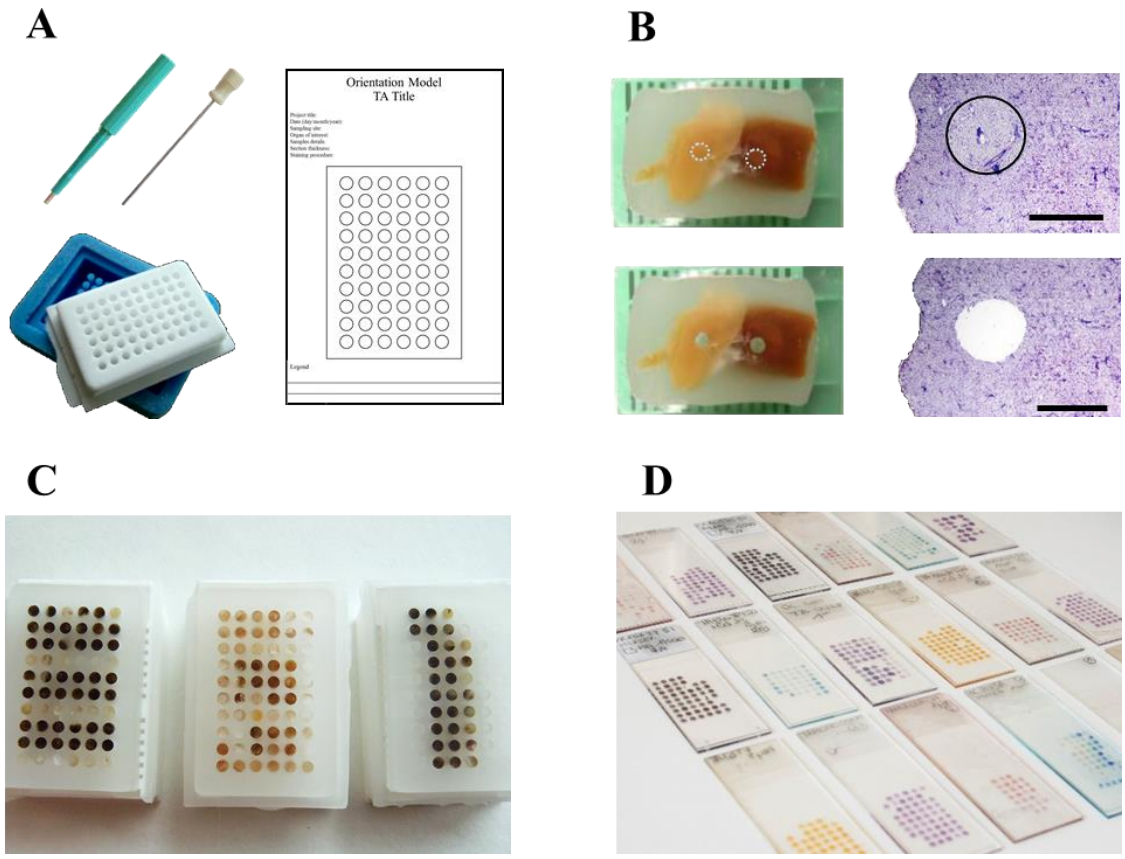
Enzyme working solution
Centrifuge the Enzyme Solution tube for 5 sec. Mix 15 µl of Enzyme Solution with 2.5 ml of Dilution Buffer.

- Pipet 20 µl of sample solution to the sample and blank 2 wells
- Pipet 20 µl of ddH_2O to the blank 1 and blank 3 wells.
- Add 200 µl of WST Working Solution to each well, and mix.
- Pipet 20 µl of Enzyme Working Solution to the sample and blank 1 wells, and mix.
- Incubate for 20 min at 37º C.
- Read the absorbance at 450 nm.
- Calculate the SOD activity (inhibition rate %) using the following equation:

SOD activity (inhibition rate %) = {[Ablank 1 - Ablank 3} - (Asample - Ablank 2)] / (Ablank 1 - Ablank 3)} x 100

2. The Tissue-Array (TA) technology

2.1. Overview of the TA procedure



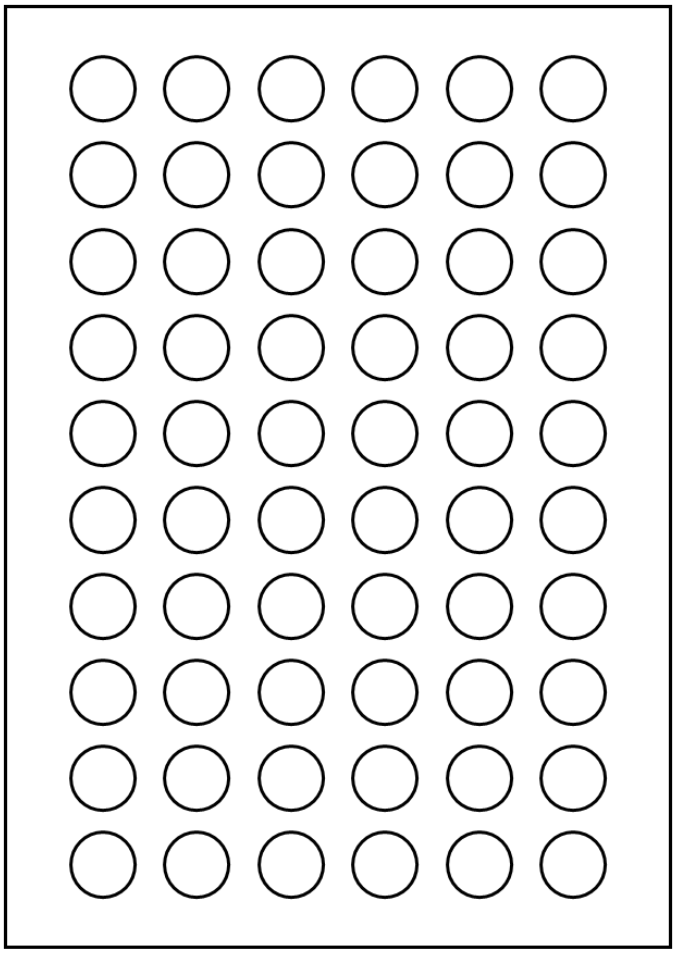
A: TA equipment showing the punching needle, silicon Array mold with frozen receptor block made from OCT medium (optimal cutting temperature compound) and Orientation Model worksheet. B: Donor blocks embedded in paraffin before and after punching with corresponding H-E micrographs; scale bar: 2 mm. C: Examples of complete TA blocks. D: TA slides after a variety of staining procedures including histological (H-E), histochemical and immunohistological staining.

TA blocks were prepared using the Array Mold® Kit (n°20015-A) containing the silicon mould suitable for paraffin embedded and frozen samples. Each TA block has a total capacity of 60 cores of 2mm diameter. Frozen or paraffin embedded donor samples are cored and transferred to the receptor block according to a clear core layout previously drawn (see Orientation Model worksheet). Cored donor tissue are brought back to storage conditions and kept available for future analysis. Complete TA blocks are cut as standard blocks in a microtome (5 µm sections for H-E staining) or a cryostat (10 µm sections for LMS test and 8 µm sections for LSC test and the assessment of intracellular accumulation of neutral lipids). Final TA blocks can be preserved at room temperature for paraffin blocks or at -80°C for frozen blocks.

2.2. Orientation Model worksheet

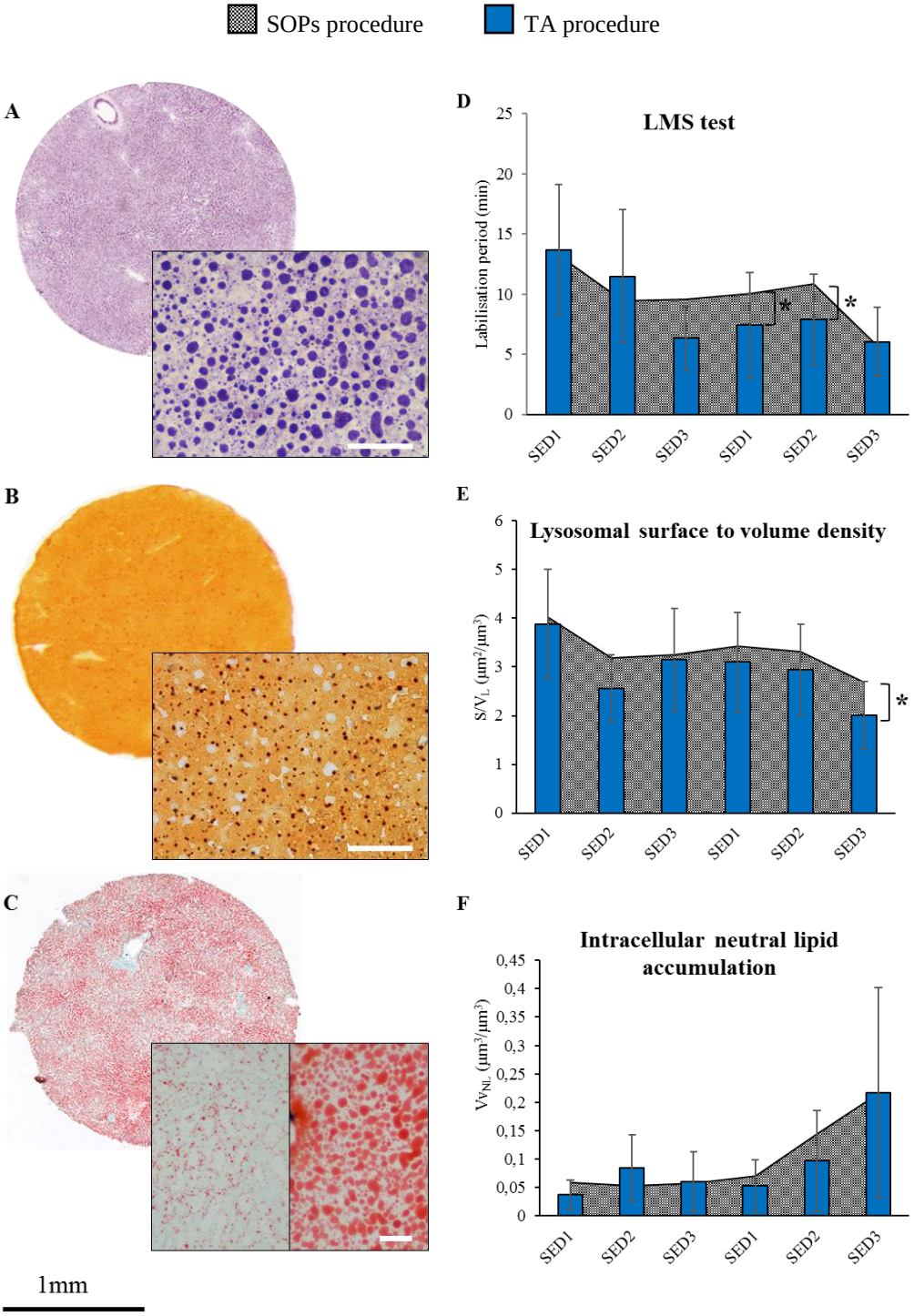
Orientation Model
TA Title

Project title:
Date (day/month/year):
Sampling site:
Organ of interest:
Samples details:
Section thickness:
Staining procedure:



Legend

2.3. The TA technology for histochemical procedures



Hepatic TA histochemical sections (8 and 10 µm) of *S. senegalensis* showing the demonstration of lysosomal enzymes activities, the acid phosphatase (A) and β-glucuronidase (B), and the accumulation of neutral lipid droplets (C). Data comparison between standard and TA procedures for lysosomal biomarkers: lysosomal membrane stability (LMS) assessed as labilisation period (D), lysosomal surface to volume ratio (S/V_L, E) and intracellular neutral lipid volume density (Vv_{NL}, E). * indicates significant differences between the two procedures ($p<0.05$); white scale: 50 µm.

3. Histochemical protocols

3.1. Lysosomal Membrane Stability (LMS) test using acid phosphatase

The determination of lysosomal membrane stability (LMS) was based on the time of acid labilisation treatment (LP, in min) required to produce the maximum staining intensity according to UNEP/RAMOGE (1999) and Broeg et al. (1999), after demonstration of acid phosphatase (AcP) activity in hepatocyte lysosomes.

3.1.1. Reagents and solutions

Sodium citrate buffer (2.5% NaCl, pH 4.5)
Dissolve 3.35 g of trisodium citrate dihydrate $C_6H_5Na_3O_7$ $2H_2O$ in 100 ml of distilled water. Set the pH at 4.5 using NaOH and HCl (at 0.1M, 1M and 5M). Add 2.5 g of sodium chloride (NaCl).

Incubation medium
Dissolve 80 mg of naphthol AS-BI-phosphatase (Sigma, N-2125) in 8ml of Dimethyl sulfoxide (DMSO). Add 392 ml of sodium citrate buffer (2.5% NaCl, pH 4.5) and 28 g of POLYPEP (Sigma, P5115).

0.1 M phosphate buffer (2.5% NaCl, pH 7.4)
Dissolve 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 19.2 ml of distilled water. Mix both solutions and set the pH at 7.4 using NaOH and HCl (at 0.1M, 1M and 5M). Add 2.5 g of sodium chloride (NaCl).
Diazonium Dye
Dissolve 0.4 g of Fast Violet in 400 ml of 0.1 M phosphate buffer (2.5% NaCl, pH 7.4).

3.0% Saline solution
Mix 24 g of NaCl in 800 ml of distilled water.

Baker buffer (2.5% NaCl, pH 7)
Neutralise (pH 7) 10 ml of formaldehyde 40% using few drops of sodium hydroxide (NaOH). Mix with 1.325 g of calcium chloride dihydrate ($2H_2O$ $CaCl_2$, 10%). Add 80 ml of distilled water and 2.5 g of sodium chloride (NaCl). The solution should be kept at 4°C.

3.1.2. Staining procedure

- Histochemical slides (10 µm) should be slowly brought to room temperature before staining.
- Prepare the incubation medium
- Place the slides in sodium citrate buffer (2.5% NaCl, pH 4.5) for 50, 40, 30, 25, 20, 15, 10, 8, 6, 4, 2 and 0 min at 37ºC.
- Incubate the samples in the incubation medium, for 20 min at 37ºC, in a shaking bath.
- Rinse the slides in saline solution (3.0%) for 5 min at 37ºC.
- Dye the sections with the Diazomium dye for 9 min at room temperature.
- Rinse the slides in tap water for 10 min.
- Fix the tissue samples in Baker buffer solution (2.5% NaCl, pH 7) for 15 min at 4ºC.
- Rinse the slides in distilled water.
- Mount the slides in Kaiser's glycerine gelatin.
- The edge of the coverslip can be sealed with nail's protector.

3.2. Lysosomal Structural Changes (LSC) using β-glucuronidase

The lysosomal structural changes (LSC) test is a quantitative technique which informs about the state of lysosomes in a cell, in response to specific environmental conditions. The visualisation of lysosomes in fish hepatocytes was based on the histochemical demonstration of β-glucuronidase activity, according to the procedure described by Moore (1976) with the modifications made by Cajaraville et al. (1989) for mussels and later adapted to fish liver by Alvarado et al. (2005).

3.2.1. Reagents and solutions

Sodium Bicarbonate
Dissolve 0.042 g of sodium bicarbonate ($NaHCO_3$) in 10 ml of distilled water. The solution should be kept at 4ºC.
0.1 M acetate buffer (2.5% NaCl, pH 4.5)
Dissolve 1.224 g of crystallized sodium acetate trihydrate ($NaCH_3COO\ 3H_2O$) (Sigma, S-8625) in 45 ml of distilled water and 0.63 ml of glacial acetic acid (CH_3COOH) in 55 ml of distilled water. This step should be undertaken in a fume cupboard. Mix both solutions and set the pH at 4.5 using NaOH and HCl (at 0.1M, 1M and 5M). Add 2.5 g of sodium chloride (NaCl). The solution should be kept at 4°C.
Incubation medium
Dissolve 28 mg of naphthol AS-BI-β-D-glucuronide (Sigma, N-1875) in 1.2 ml of 50 mM sodium bicarbonate. Add 98.8 ml of 0.1 M acetate buffer (2.5% NaCl, pH 4.5). Add 15 g of polyvinyl alcohol (Sigma, P-8136). This last step should be undertaken using a hot plate with continuous stirring. The incubation medium should be kept in a shaking bath at 37ºC until the target temperature is reached.
Saline solution (2.5%)
Dissolve 2.5 g NaCl in 100 ml of distilled water. The solution should be kept in a shaking bath at 37ºC.
Post-coupling medium
Dissolve 0.04 g of Fast Garnet GBC (Sigma, F-8761) in 40 ml of 0.1 M phosphate buffer (2.5% NaCl, pH 7.4). The solution is stable for only few hours at 4°C and should be used immediately.
0.1 M phosphate buffer (2.5% NaCl, pH 7.4)
Dissolve 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 19.2 ml of distilled water. Mix both solutions and set the pH at 7.4 using NaOH and HCl (at 0.1M, 1M and 5M). Add 2.5 g of sodium chloride (NaCl).
Baker buffer (2.5% NaCl, pH 7)
Neutralise (pH 7) 10 ml of formaldehyde 40% using few drops of sodium hydroxide (NaOH). Mix with 1.325 g of calcium chloride dihydrate ($2H_2O\ CaCl_2$, 10%). Add 80 ml of distilled water and 2.5 g of sodium chloride (NaCl). The solution should be kept at 4°C.

3.2.2. Staining procedure

- Prepare the incubation medium and the saline solution.

- Histochemical slides (8 µm) should be slowly brought to room temperature before staining.
- Incubate the samples in the incubation medium for 20 min at 37ºC, in a shaking bath.
- Prepare the Post-coupling medium. The solution should be protected from the light with constant stirring.
- Rince the slides in the saline solution (2.5%) for 2 min at 37ºC, in a shaking bath.
- Transfer the slides to the Post-coupling medium for 10 min at room temperature, in the dark and with constant shake.
- Fix the tissue samples in Baker buffer solution (2.5% NaCl, pH 7) for 10 min at 4ºC.
- Rinse the slides in distilled water.
- Mount the slides in Kaiser's glycerine gelatin.
- The edge of the coverslip can be sealed with nail's protector.

3.3. Oil Red O staining

3.3.1. Reagents and solutions

Baker buffer (2.5% NaCl, pH 7)
Neutralise (pH 7) 10 ml of formaldehyde 40% using few drops of sodium hydroxide (NaOH). Mix with 1.325 g of calcium chloride dihydrate ($2H_2O$ $CaCl_2$, 10%). Add 80 ml of distilled water and 2.5 g of sodium chloride (NaCl). The solution should be kept at 4°C.
Oil Red O solution
Dissolve 0.25 g of 0.5% ORO in 50 ml of isopropilic alcohol. Add 33.3 ml of distilled water (dilution 3:2). Mix the solution for 15 min. Filter the dye and protect from the light and use in the next couple of hours.
Isopropilic alcohol (60%)
Mix 60 ml of isopropilic alcohol with 40 ml of distilled water without stirring. The solution should be kept in the fridge and covered with film to avoid its evaporation.

3.3.2. Staining procedure

- Fix the tissue samples (8 µm) in Baker solution (2.5% NaCl, pH 7) for 15 min at 4ºC.
- Dry the sections in the air.
- Rinse the sections in isopropilic alcohol (60%).
- Dye the sections in Oil Red O solution for 20 min at room temperature.
- Transfer the slides to isopropilic alcohol (60%) for 1 min.
- Counterstain the sections in 0.1% Fast Green FCF dye for 30 min.
- Rinse the slides in distilled water.
- Mount the slides in Kaiser's glycerine gelatin.
- The edge of the coverslip can be sealed with nail's protector.

4. Histological protocols

4.1. Fixative solutions for histology

4% neutral buffered formol, pH 7.2:

57.84g $Na_2HPO_4 \cdot H_2O$ + 5.12g $NaH_2PO_4 \cdot H_2O$ + 200ml of Formaldehyde (37%), to be completed with dH_2O (Vol_{Tot}: 2 l). Samples fixed in formalin should be kept in the solution at 4ºC for 24 hr. They can be preserved in 70% ethanol.

Bouin's solution:

100 ml of Formaldehyde (37%) + 70 ml of acetic acid + acid picric (at saturation) + 830 ml dH_2O. Samples fixed in Bouin should be kept in the solution at 4ºC for 24 hr. They should then be transferred in 8% formic acid at room temperature for 24hr. They can be preserved in 70% ethanol.

4.2. Tissue processing

After fixation, samples are dehydrated following procedure as below, before being embedded in paraffin:

70% ethanol	1 hr
96% ethanol	1 hr
96% ethanol	1 hr
100% ethanol	1 hr
100% ethanol	1 hr
IMS	1 hr
Xylene	1 hr
Xylene	1 hr
Paraffin	2 hr
Paraffin	2 hr
Paraffin	2 hr

IMS: ratio 1:1 100% ethanol and Xylene

4.3. Hematoxylin-eosin (H-E) staining

Xylene	10 min
Xylene	10 min
100% ethanol	2 min
100% ethanol	2 min
96% ethanol	2 min
70% ethanol	2 min
dH_2O	5 min
Hematoxylin	4 min
dH_2O	4 min
Acid alcohol	10 s
dH_2O	5 min
Lithium carbonate	10 s
dH_2O	1 min
Eosin	1 min 30 sec
dH_2O	5 sec
dH_2O	1 min 30 sec
70% ethanol	5 sec
96% ethanol	10 sec
100% ethanol	15 sec
100% ethanol	20 sec
Xylene	1 min
Xylene	1 min

5. Characterisation of the reproductive cycle

5.1. Male gamete developmental stages

Gamete developmental stages in males were determined according to García-López et al. (2006) and were classified into the following stages:

- Immature (or resting). The seminiferous lobules are forming. SPG and SPC can be detected at this stage.

- Stage I. Early spermatogenesis. This stage is characterised by the presence of numerous germinal cysts in the testis cortex containing SPG. It is possible to identify few SPD in the lumen of seminiferous lobules and some SPD and SPZ in the medullar efferent ducts. Empty spaces can be observed in the cortex and the medullar areas.

- Stage II. Mid spermatogenesis. At this stage, germ cells at all developmental stages can be observed, from SPG to SPZ. In the cortex, the number of SPG is decreasing as they differentiate into SPC (initiation of the meiosis). In comparison with the previous stage, no empty spaces are observed in the seminiferous lobule. Instead, the small central lumen is filled with SPD. It is possible to identify some SPD and SPZ in the medullar efferent ducts.

- Stage III. Late spermatogenesis. The number of SPC in germinal cysts decreases and SPD become the main cell type. They are found in the lumen of the seminiferous lobule (semi-cystic spermatogenesis). More SPZ are observed in the medullar efferent ducts.

- Stage IV. Functional maturation. SPD are becoming less abundant in the cortex as they are differentiated into SPZ, which accumulate in the lumen of the medullar efferent ducts. SPG associated with Sertoli cells can be identified in the cortex.

- Stage V. Recovery (or post-spawning). The number of SPD in the cortex and SPZ in the lumen of the medullar efferent ducts decreases. Numerous SPG and Sertoli cells can be observed in the distal part of the cortical seminiferous lobules showing the initiation of germ cell proliferation.

5.2. Female gamete developmental stages

Gamete developmental stages in females were determined according to Murua and Motos (2006) and were classified into four categories, based on the frequency of each oocyte phase detected in the gonads:

- Stage I. Growth (or pre-vitellogenesis). The growing oocyte progress from a Central Nucleolar Stage (1) with a small nucleus and a unique and central nucleolus to a Perinuclear Nucleoli Stage (S1) with a larger nucleus, larger ooplasm and several nucleoli placed in the periphery of the nucleus. Several types of germ cells can be detected at this stage including oogonia and S1 oocytes, cortical alveolar oocytes (S2), late cortical alveolar oocytes (S3) and early vitellogenic oocytes (S4) (Figure 5A).

- Stage II. Early vitellogenesis. This stage is characterised by the presence of S5 oocytes, which are bigger in size and show a wide zona radiata, small yolk inclusions and lipid vesicles. The nucleus of S5 oocytes is central and surrounded by several nucleoli located at its periphery.

- Stage III. Late vitellogenesis. S6 oocytes can be identified at this stage (Figure 5B). They typically present a larger zona radiata, lipid granules and yolk granules occupying most of the ooplasm. At this stage, the presence of the theca and granular layer is indicative of the development of the follicular layer. The nucleus starts migrating towards the animal pole and the nuclear membrane starts breaking. Atretic oocytes may be detected at this stage.

- Stage IV. Maturation. Mature female gonads are characterised by the presence of S7 oocytes showing a migrating nucleus towards the animal pole and nuclear membrane breakage. Atretic oocytes may be detected at this stage.

6. Histopathological guidelines

6.1. Categories of hepatic histopathological lesions in flatfish species (modified after Feist et al., 2004) and scoring system used for their quantification in sole adapted from Lang et al., 2006.

Lesion categories	Lesions	Lesion stages	Lesion scores
Non-specific lesions	Inflammatory changes (infiltration, granulomatosis)		
	Degenerative changes (coagulative or single cell necrosis, atrophy)		
	Proliferative changes (hepatocellular regeneration, fibrosis, bile duct hyperplasia)		
	Increased number/area of macrophage aggregates	Mild	1
	Lipidosis	Medium	2
	Parasites	Severe	3
Early non-neoplastic toxicopathic lesions	Hepatocellular/nuclear pleomorphism		
	Hydropic vacuolation (hepatic/biliary/pancreatic)		
	Phospholipidosis of hepatocytes	Mild	4
	Fibrillar inclusions	Medium	5
	Peliosis and spongiosis hepatis	Severe	6
Foci of cellular alteration	Clear cell Vacuolated		
	Eosinophilic	Mild	7
	Basophilic	Medium	8
	Mixed	Severe	9
Benign neoplasms	Hepatocellular adenoma Cholangioma		
	Haemangioma	Mild	10
	Pancreatic acinar cell adenoma	Medium	11
	Other	Severe	12
Malignant neoplasms	Hepatocellular carcinoma Cholangiocarcinoma		
	Pancreatic acinar cell carcinoma	Mild	13
	Haemangiocarcinoma	Medium	14
	Other	Severe	15

6.2. Categories of gill histopathological lesions in flatfish species and scoring system used for their quantification in sole adapted from Bernet et al., 1999.

Lesion categories	Functional unit	Lesions	w
Circulatory disturbances		Haemorrhage/Hyperaemia/ aneurysm	1
		Intercellular oedema	1
Regressive changes	Epithelium	Architectural and structural alterations	1
		Plasma alterations	1
		Deposits	1
		Nuclear alterations	2
		Atrophy	2
		Necrosis	3
	Supporting tissue	Architectural and structural alterations	1
		Plasma alterations	1
		Deposits	1
		Nuclear alterations	2
		Atrophy	2
		Necrosis	3
Progressive changes	Epithelium	Hypertrophy	1
		Lamellar fusion	1
		Hyperplasia	2
	Supporting tissue	Hypertrophy	1
		Hyperplasia	2
Inflammatory responses		Exudate	1
		Activation of RES	1
		Infiltration	2
Tumours (neoplasms)		Benign tumour	2
		Malignant tumour	3

w: lesion importance factor.

6.3. Categories of liver histopathological lesions in flatfish species and scoring system used for their quantification in sole adapted from Bernet et al., 1999.

Lesion categories	Functional unit	Lesions	w
Circulatory disturbances		Haemorrhage/Hyperaemia	1
		Intercellular oedema	
Regressive changes	Liver tissue	Architectural and structural alterations	1
		Plasma alterations	1
		Deposits	1
		Nuclear alterations	2
		Atrophy	2
		Necrosis	3
		Vacuolar degeneration	
	Interstitial tissue	Architectural and structural alterations	1
		Plasma alterations	1
		Deposits	1
		Nuclear alterations	2
		Atrophy	2
		Necrosis	3
	Bile duct	Architectural and structural alterations	1
		Plasma alterations	1
		Deposits	1
		Nuclear alterations	2
		Atrophy	2
		Necrosis	3
Progressive changes	Liver tissue	Hypertrophy	1
		Hyperplasia	2
	Interstitial tissue	Hypertrophy	1
		Hyperplasia	2
	Bile duct	Hypertrophy	1
		Hyperplasia	2
		Wall proliferation of bile ducts	
Inflammatory responses		Exudate	1
		Activation of RES	1
		Infiltration	2
Tumours (neoplasms)		Benign tumour	2
		Malignant tumour	3

w: lesion importance factor; RES: Reticuloendothelial system.

6.4. Categories of gonad histopathological lesions in flatfish species and scoring system used for their quantification in sole based on Cuevas et al., 2015a and adapted to the classification provided by Bernet et al., 1999.

Lesion categories	**Lesions**	**w**
Circulatory disturbances	Haemorrhage/Hyperaemia	1
Regressive changes	Architectural and structural alterations	1
	Plasma alterations	1
	Deposits	1
	Nuclear alterations (pyknotic oocytes/spermatocytes)	2
	Atresia	3
	Intersex	3
	Necrosis	3
Progressive changes	Hypertrophy	1
	Hyperplasia	2
Inflammatory responses	Exudate	1
	Activation of RES	1
	Granulomatosis	2
	Infiltration	2
Tumours (neoplasms)	Benign tumour	2
	Malignant tumour	3

w: lesion importance factor.

Une Onde Sous Celle d'un Pinceau
Fragile, Suspendue, une Esquisse à l'Aquarelle
Prête à Rompre, Comme un Appel
De l'Océan, un Soupçon d'Eau

C.B. MMXV