PhD Thesis

Integrated assessment of environmental pollution using diverse sentinel organisms within Basque marine environments

Presented by

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To accomplish great things, we must
not only act, but also dream;
not only plan, but also believe.

Anatole France

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Scientific contributions

The results from different works carried out during the development of this thesis have led to a series of presentations in international congresses and publications in different peer-reviewed journals. Some of them represent the main core of the present thesis; others have served to give context to the followed lines of work. A number of them have already been published and others are accepted for publication. They are listed below according to the Chapters of this thesis:

Chapter 1

Cuevas, N., Larreta, J., Rodríguez, J.G., Zorita, I., 2011. A visual guideline for the determination of imposex in *Nassarius reticulatus* and *Nassarius nitidus*. Rev. Invest. Mar. AZTI-Tecnalia 18, 134–152.

Cuevas, N., Belzunce-Segarra, M.J., Borja, Á., Franco, J., Garcia Alonso, J.I., Garmendia, J.M., Larreta, J., Muxika, I., Rodriguez, J.G., Sariego, C., Solaun, O., Valencia, V., Zorita, I., 2011. Advantages and disadvantages of different methodologies for evaluating tributyltin (TBT) pollution and its effects under the European Water Framework Directive. Poster, ECSA, Bordeaux.

Cuevas, N., García Alonso, J.I., Larreta, J., Rodríguez, J.G., Sariego, C., Zorita, I., 2014. Monitoring the effectiveness of the European tributyltin regulation on the Basque coast (northern Spain) by assessing imposex in two gastropod species (*Nassarius reticulatus* and *Nassarius nitidus*). Chem. Ecol. 30, 636–642.

Chapter 2

Cuevas, N., Costa, P.M., Franco, J., Larreta, J., Zorita, I., 2014. Development of histopathological condition indices in digestive gland and gonad of mussels (*M. galloprovincialis*): Effects of season and other potential confounding factors. Platform, ECSA, Sesimbra.

Cuevas, N., Zorita, I., Costa, P.M., Franco, J., Larreta, J., 2015. Development of histopathological indices in the digestive gland and gonad of mussels: Integration with contamination levels and effects of confounding factors. Aqua. Toxicol. 162, 152–164.

Chapter 3

Cuevas, N., Franco, J., Larreta, J., Zorita, I., 2012. Biomarkers survey in contaminated estuarine habitats of Nervión estuary in two fish species: *Pomatoschitus sp*. and *Solea solea*. Poster, ESCPB, Bilbao.

Cuevas, N., Franco, J., Larreta, J., Zorita, I., 2013. Monitoring contaminants and biomarker responses in two fish species (*Pomatoschistus* sp. and *Solea solea*) in the Ibaizabal estuary (N. Spain). Platform, PRIMO, Faro.

Cuevas, N., Franco, J., Larreta, J., Zorita, I., 2014. Health status of gobies (*Pomatoschistus* sp.*)* collected along the Ibaizabal estuary (SE Bay of Biscay). Platform, ISOBAY, Bordeaux.

Cuevas, N., Zorita, I., Franco, J., Costa, P.M., Larreta, J., accepted. Multi-organ histopathology in gobies for estuarine environmental risk assessment: A case study in the Ibaizabal estuary (SE Bay of Biscay). Estuar. Coast. Shelf Sci.

Chapter 4

Garmendia, J.M., **Cuevas, N.**, Larreta, J., Zorita, I., Quincoces, I., 2013. Efectos de la contaminación en peces y presencia de basuras marinas en los fondos de la plataforma costera vasca: Aportaciones para la Directiva Marco de la Estrategia Marina. Rev. Invest. Mar. AZTI-Tecnalia 20, 164–190.

Zorita, I., **Cuevas, N.**, 2014. Protocol for fish disease assessment in marine environmental monitoring using common sole (*Solea solea*, Linnaeus 1758) as sentinel organism: Identification of externally visible diseases and liver histopathology. Rev. Invest. Mar. AZTI-Tecnalia 21, 1–18.

Cuevas, N., Costa, P.M., Larreta, J., Quincoces, I., Zorita, I., 2014. Monitoring biological effects of contaminants in sole (*Solea solea*) and hake (*Merluccius merluccius*) from the Basque continental shelf. Poster, ISOBAY, Bordeaux.

Cuevas, N., Zorita, I., Costa, P.M., Quincoces, I., Larreta, J., Franco, J., 2015. Histopathological indices in sole (*Solea solea*) and hake (*Merluccius merluccius*) for implementation of the European Marine Strategy Framework Directive along the Basque continental shelf (SE Bay of Biscay). Mar. Pollut. Bull. 94, 185–198.

Chapter5

Cuevas, N., Zorita, I., Costa, P.M., Larreta, J., Franco, J., 2015. Histopathological baseline levels and confounding factors in sole for marine risk assessment. Platform, CICTA, Vila Real.

Cuevas, N., Zorita, I., Costa, P.M., Larreta, J., Franco, J., accepted. Histopathological baseline levels and confounding factors in common sole (*Solea solea*) for marine environmental risk assessment. Mar. Environ. Res.

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Acronyms and abbreviations

AAS: Atomic Absorption Spectometry

AChE: Acetylcholinesterase

AFS: Anti-Fouling System Agreement

ANOVA: Analysis of variance

ASE: Accelerated Solvent Extraction

BCR: Community Bureau of Reference

BDI: Butyltin Degradation Index

BELQUAM: Biological Effects Quality Assurance in Monitoring Programmes

BFRs: Brominated Flame Retardants

BSC: Bucharest Convention in the Black Sea

CBs: Chlorinated Biphenyls

Cd: Cadmium

CEMP: Coordinated Environmental Monitoring Programme

CPF: Concentric Periductal Fibrosis

Cr: Chromium

CRM: Certified Reference Material

Cu: Copper

CYP1A: Cytochrome P4501A

DBT: Dibutyltin

DDTs: Organochloride pesticides

DPSIR: Driver - Pressure - State - Impact - Response

dw: Dry weight

EC: European Community

EEA: European Environment Agency

EEZ: Exclusive Economic Zone

e.g.: For example

EPA: Environmental Protection Agency

EQRs: Ecological Quality Ratios

EQSs: Environmental Quality Standards

ERA: Environmental Risk Assessment

EROD: Ethoxyresorufin-O-deethylase

EU: European Union

F: Female

Fe: Iron

FV: Fat Vacuolation

GC-ICP-MS: Gas Chromatography - Inductively Coupled Plasma Analysis - Mass Spectrometry

GC-MS: Gas Chromatography - Mass Spectrometry

GES: Good Environmental Status

GESAMP: Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection

GI: Gonad Index

GPC: Gel Permeation Chromatography

GSI: Gonadosomatic Index

H&E: Haematoxylin and Eosin

HCHs: Hexachlorocyclohexane

HCl: Hydrochloric acid

HELCOM: Helsinki Convention in the Baltic Sea

Hg: Mercury

HNO_3: Nitric acid

HNP: Hepatocellular Nuclear Pleomorphism

HP: Hyperplasia

HSI: Hepatosomatic Index

HT: Hypertrophy

HV: Hydropic Vacuolation

I%: Perfectage of imposex-affected females

ICES: International Council for Exploration of the Seas

i.e.: Id est

IMO: International Maritime Organization

K: Condition factor

LDPSA: Laser Diffraction Particle Size Analyzer

LOEs: Lines of Evidence

M: Male

MBT: Monobutyltin

MEDPOL: Barcelona Convention in the Mediterranean Sea

$MgCl_2$: Magnesium chloride

MMCs: Melanomacrophage Centers

MSFD: Marine Strategy Framework Directive

n: Sample number

n.d.: No data

Ni: Niquel

nm: Nautical mile

n.o.: Not observed

NRC: National Research Council of Canada

OSPAR: Oslo and Paris Convention in the North-Atlantic Ocean

***p*:** p-value

PAHs: Polycyclic Aromatic Hydrocarbons

Pb: Lead

PCA: Principal Components Analysis

PCBs: Polychlorinated Biphenyls

PEL: Probable Effects Level

POPs: Persistent Organic Substances

R: Spearman's rank-order

RNA: Ribonucleic Acid

RPLI: Relative Penis Length Index

RSCs: Regional Sea Conventions

S%: Percentage of sterile females

SE: South-East

SGIMC: Study Group on Integrated Monitoring of Contaminants and Biological Effects

Sn: Tin

SQGs: Sediment Quality Guidelines

SQG-Qs: Sediment Quality Guidelines Quotiens

TBT: Tributyltin

TEL: Threshold Effects Level

THGA: Transversely Heated Graphite Atomizer

TOM: Total Organic Matter

TPT: Triphenyltin

UEZ: Uraren Esparru Zuzentaraua

UNEP: United Nations Environment Programme

UV-B: Ultraviolet B

VDS: Vas Deferens Sequence

VDSI: Vas Deferens Sequence Index

w: Weight

WFD: Water Framework Directive

WGBEC: Working Group on Biological Effects of Contaminants

WOE: Weight of Evidence

ww: Wet weight

Zn: Zinc

Laburpena

Kutsadura ingurune urtarren osasun egoera mehatxatzen duen ingurumen arazorik garrantzitsuenetariko bat da. Hau dela eta, sistema urtarretan kutsatzaileek sortzen dituzten efektuak erregulatu, arindu eta berreskuratzeko helburuekin kutsaduraren inguruko zuzentarau nazional eta internazional desberdinak garatu dira. Tesi honetako ***Sarrera orokorrean*** islatu den moduan, Europako herrialdeetako araudi nabarmenetarikoen artean Uraren Esparru Arteztaraua (UEA) eta Itsas Estrategiaren Arteztaraua aurkitzen dira. Bi arteztarau hauen helburu nagusia itsasoko uren, habitaten eta baliabideen egoera osasuntsua mantentzeko edota lortzeko ingurumen urtarren kudeaketarako baliagarriak eta egokiak diren teknika eta planteamenduak bereganatzea da. Azken hau lortzeko asmoarekin, itsas ingurumeneko kutsaduraren azterketa integratzailea abiapuntutzat hartuz, teknika kimikoen eta osasun egoera islatzen duten efektu biologikoen tresnen garapena ikertu behar da.

ICES/OSPAR SGIMC lan taldeak Europako itsas ingurumenaren kutsadura aztertzen du, kutsatzaileen eta efektu biologikoen ebaluaketa integratzailean oinarritutako ikerketak burutzen dituelarik. Hori dela eta, tesi-lan honetan SGIMC taldeak proposatutako eskema integratzailea jarraitzen da. Ikuspuntu integratzaile hau matrize natural desberdinen (*i.e.* ura, sedimentu eta biota) kutsadura mailen eta hauek organismo behaleetan sortarazten dituzten efektu biologikoen aldibereko neurketetan oinarritzen da. Horregatik, ingurumenaren kutsaduraren ebaluaketa integratzaile baten bitartez, ehun eta sedimentuen kimikarekin batera, *imposex* edota histopatologia erabiliz, tributileztainuaren agerpenaren edota organismo behale desberdinen (*i.e.* gastropodo, muskuilu eta arrainak) osasun egoera orokorra aztertu da. Horrela, tesi honen helburu espezifikoak bost kapitulu desberdinetan zehar aurkeztu dira.

Lehenengo Kapituluan, tributileztainuarekin egindako *antifouling* margoen erabilpena erregulatzen duen Europako araudiaren eraginkortasuna ikertu zen. Horretarako, 2011an Euskal Herriko kostaldean lagindutako bi gastropodo espezien (*Nassarius reticulatus* eta *Nassarius nitidus*) *imposex* eta organoeztainuaren bioakumulazio mailak zehaztu ziren. Ondoren, emaitza hauek 2007an (tributileztainuaren debekuaren urtebete aurretik) burututako hasierako ikerketan lortutako mailekin konparatu ziren. Emaitzek laginketa puntu gehienetan 2011n neurtutako *imposex* mailak 2007koak baino txikiagoak zirela adierazi zuten. Era berean, 2011an *N. reticulatus*en emeetan tributileztainuaren bioakumulazio maila baxuagoak aurkitu ziren. Hala ere, butileztainuaren degradazioa indizeak laginketa puntu batzuetan tributileztainuaren sarrera berriak islatu zituen. Laburbilduz, tributileztainuarekin egindako margoak debekatzen dituen lege-neurriak konposatu organiko honek sortarazten dituen efektu biologikoak murriztea lortu du. Oraindik, alabaina, leku batzuetan konposatu honen presentzia bistakoa denez, gai honen inguruan ikerketa gehiago beharrezkoa da.

Bigarren Kapituluan, histopatologia erabili zen kutsadura maila desberdinak (konposatu organiko eta metalikotan) zituzten bost laginketa puntutik jasotako muskuiluen (*Mytilus galloprovincialis*) osasun egoera ikertzeko. Horrez gain, kapitulu honetan muskuiluen liseri guruinaren eta gonadaren osasun egoera aztertzeko histopatologian oinarritutako indize semi-kuantitatiboak garatu ziren. Aztertutako hogeita hiru kalte histopatologikoak indize bitan integratu ziren, bat liseri guruinarako eta bestea gonadarako. Modu honetan, muskuiluen osasun egoera hobeto ulertu genuen. Emaitzek, gehien kutsatutako laginketa puntuetan hartutako muskuiluek ehun efektu kaltegarri nabarmenenak jasan zituztela adierazi zuten. Gutxi edota erdi kutsatutako lekuetan, berriz, kutsadura mailak ez zuen ehunetan kalte esanguratsurik sortu. Hala ere, parasitoek, gutxi kutsatutako laginketa puntuetatik hartutako muskuiluen indize histopatologikoak gehiegi balioetsi (*i.e.* ehun kalte larriagoa adieraziz) zituzten. Oso kutsatutako lekuetatik jasotako muskuiluetan, ostera, guztiz kontrakoa gertatu zen. Aldi berean, laginketa puntuen arteko desberdintasun adierazgarrienak udazkenean agertu ziren, heldutasun fasean efektu fisiologiko naturalek erantzun histologikoak ez dituztelako oztopatzen. Beraz, nahaste faktoreen (*i.e.* sasoiko aldaketak eta parasitoak) gorabeherak kontu handiz aztertzen badira, liseri guruinaren eta gonadaren indize histopatologikoek muskuiluen osasun egoera ikertzeko sentikortasun egokia islatuko dute.

Hurrengo bi kapituluetan, arrain espezie desberdinak erabiliz itsasoaren osasun egoera orokorra aztertu zen. ***Hirugarren Kapituluan***, Ibaizabal estuarioko (Bizkaiko Golkoko hegoekialdean) ingurumenaren arriskuak ebaluatzeko helburuarekin, gobioetan (*Pomatoschistus* spp.) organo anitzeko (gibela, zakatzak, giltzurruna eta gonada) histopatologiaz gain, metalen bioakumulazio eta sedimentuen kutsadura mailak ikertu ziren. Horretarako, hiru urtetan zehar Ibaizabal estuarioko lau trantsektu aztertu ziren. Emaitzek, sedimentuak, metal eta konposatu organikotan, oso edota erdi kutsatuak zeudela islatu zuten. Beraz, efektu biologiko kaltegarriak agertzea litekeena zen. Era berean, estuarioan zehar jasotako gobioen metalen bioakumulazio mailek eta organo anitzeko indize histopatologikoek antzeko eritasun mailak erakutsi zituzten. Hala ere, arrainen osasun egoera hobea nabarmenduz, azkeneko urtean (2013) bai metalen bioakumulazio mailak bai indize histopatologikoak murriztu ziren. Horrez gain, kutsatzaileen detoxifikazio prozesuak azpimarratuz, gibelak, zakatzek eta giltzurrunak barea eta gonada baino kalte histopatologiko esanguratsuagoak erakutsi zituzten. Hepatozitoen lipidoen bakuolazioa gibelean, lamelen fusioa zakatzetan eta melanomakrofagoen zentruak giltzurrunean, Ibaizabaleko gobioetan nagusitu ziren kalteak dira. Aldaketa histopatologiko hauek kutsatzaile ez espezifikoen presentzia islatzen dute, nahiz eta beste ingurumeneko faktoreen eragina ezin den baztertu.

Laugarren Kapituluan, Itsas Estrategiaren Arteztarauaren 8. Deskriptoreari erantzuna emateko eta arrain histopatologiaren erabilera balioztatzeko asmoz, mihi-arrain arrunta (*Solea solea*) eta legatza europarra (*Merluccius merluccius*), sedimentuekin batera, Euskal Herriko plataforma kontinentalean zehar bi kanpainetan lagindu ziren. Hauetatik, arrain behale egokia zein den aztertu zen eta hauetan, jada existitzen ziren bi indize histopatologiko (Costa et al. 2009 *versus* Lang et al. 2006) aplikatu eta konparatu ziren. Azterketa honen arabera, metalen bioakumulazio mailak eta gibeleko lesio histopatologikoak mihi-arrain arruntean legatza europarrean baino nabarmenagoak izan ziren. Ondorioz, mihi-arrain arrunta, seguru aski bere portaera bentonikoagatik, legatza europarra baino arrain behale sentikorragoa dela esan daiteke. Bestalde, bi indize histopatologikoen konparaketak, hauen egokitasuna eta zehaztasuna azpimarratzeaz gain, bi aplikazioak (Costa et al. 2009; Lang et al. 2006) bai gibelean bai gonadan oso korrelazionatuta zeudela agerian utzi zuen. Gainera, arrain histopatologiaren arabera, bi espezieen gibelean eta gonadan osasun egoeraren aldaketa txikiak erregistratu ziren, kalte arinak (*i.e.* ez espezifiko eta ez neoplasiko toxikopatiko goiztiarrak) behatuz. Emaitza hauek Euskal Herriko plataforma kontinentaletik jasotako sedimentuen kutsadura mailekin (metalekin erdi kutsatua eta konposatu organikoekin ez kutsatua) bat zetozen. Hala ere, sedimentuen kutsadura, metalen bioakumulazio eta indize histopatologikoen arteko korrelazio ezak, kutsaduraz gain, analizatutako efektu biologikoen eragileak aztertu beharreko beste faktore batzuk izan daitezke. Labur esanda, arrain histopatologia Euskal Herriko plataforma kontinentalaren ingurumenaren kutsaduraren indikatzaile egokia izan daiteke. Hala ere, itsas zabaleko ingurumeneko organismo behaleen ebaluaketa histopatologiakoaren inplementazioa lortzearren, oinarrizko mailen eta nahasketa faktoreen gorabeherak zehaztea ezinbestekoa da.

Ondorioz, ***Bosgarren Kapituluan*** gibeleko eta gonadako azterketa histopatologikoa egiteaz gain, parametro biometriko eta metalen bioakumulazio mailak aztertu ziren gibelean hilero, urte oso batez, Euskal Herriko plataforma kontinentalean (erreferentzi baldintzekin) arrantzatutako mihi-arrain arruntetan. Emaitzetan, Zn bioakumuazio maila altuak aurre-errutean dauden emeetan nagusitu ziren; Cd, Cu eta Pb bioakumulazio mailetan, berriz, bi sexuetan sasoiko bioakumulazio eredua frogatu zen. Hala ere, azken datu hauek kontuz interpretatu behar dira, batez ere biologia eta ingurumenaren aldaketak kontuan hartzen ez direnean. Horrez gain, sasoiko aldaketek eta generoak nahasketa faktore legez jokatu dezakete, beraz, bai laginketa momentua bai generoa laginketa diseinatzean eta datuen interpretazioan ez dira ahaztu behar. Hori dela eta, parametro hauek ikertzeko garai egokiena gametogenesiaren fase goiztiarretan (ekainetik irailera) dela dirudi. Hala ere, nahiz eta alterazio histopatologikoen prebalentziak gibelean sasoiko aldaketak erakutsi, mihi-arrain arrunten gibeleko indize histopatologikoa erabiltzen zenean, urtaroa, generoa eta adina ez ziren nahaste faktoretzat hartu. Azken hau, *biomonitoring* helburuetarako onuragarria izan daiteke.

Azkenik, ***Eztabaida orokorrean***, Kapitulu desberdinetan lortutako emaitzak, aurretik zehaztutako helburuei erantzunez, ikuspuntu integratzaile batetik analizatu dira. Hitz gutxitan, lan honetan ingurumeneko kutsaduraren ebaluaketa integratzailea burutu da, zeinetan organismo behale desberdinetako efektu biologikoak eta sedimentu edota ehunetako kimika konbinatu diren, itsas ingurumenaren osasun egoera ikertzeko planteamendu aproposa dela baieztatuz. Hala ere, datuen interpretazioa egokia lortzeko asmoz, nahasketa faktoreen eragina eta ingurumen ebaluaketaren irizpideak zehaztearen garrantzia azpimarratu behar da. Ondorioz, ingurumeneko zuzentarauen helburuak betetzeko (adib. Itsas Estrategia Arteztarauaren 8. Deskriptorea) ebaluaketa integralaren bitartez erantzun bateratu eta osoago bat lortu daitekela frogatu da.

Summary

The pollution of aquatic environments is one of the most significant environmental dilemmas that threats to the aquatic health status. This environmental problem has therefore led to the development of different national and international directives in order to regulate, mitigate and restore the contaminant effects on aquatic systems. As it is remarked in the ***General introduction*** of this thesis, among these legislative regulations, the Water Framework Directive (WFD) and the Marine Strategy Framework Directive (MSFD) are the most relevant policies within European regions. The principal objective of both directives is to provide a valuable and appropriate methodology for the management of the aquatic systems with the aim of achieving and/or maintaining a "good status" of marine waters, habitats and resources on the basis of an ecosystem-based approach. For this purpose, it is crucial to develop not only chemical tools, but also biological effects methods for the determination of aquatic health status within an integrated assessment of environmental pollution.

The present work follows the scheme proposed by the ICES/OSPAR SGIMC (Study Group on Integrated Monitoring of Contaminants and Biological effects) for the integrated assessment of environmental pollution in European Regional Seas. This integrated approach is based on the combination of simultaneous measurements of contaminant levels in diverse matrices (*i.e.* water, sediment and biota) and their respective biological effects in different sentinel organisms. Thus, the present work attempts to address the evaluation of an integrated assessment of environmental pollution using imposex or histopathology, in combination with tissue and/or sediment chemistry, for assessing tributyltin exposure or general health status in a set of sentinel organisms (*i.e.* gastropods, mussels and fishes) within the Basque marine environments. The specific aims of this thesis are presented in five different Chapters.

Chapter 1 aimed to assess the effectiveness of the European regulation on the use of tributyltin-based antifouling paints. For that, imposex and organotin bioaccumulation levels were determined in two gastropod species (*Nassarius reticulatus* and *Nassarius nitidus)* collected along the Basque coast during 2011 and results were compared with the levels recorded in an initial survey in 2007 (one year before the TBT ban). Results indicated that imposex levels in 2011 were lower than those determined in 2007 at most of the sampling sites. In agreement, TBT content in the female body burden of *N. reticulatus* presented a lower maximum content than in 2007. However, the butyltin degradation index suggested recent TBT inputs in some of the sampling sites. In brief, the legislative measure is contributing to the reduction of TBT-induced biological effects along the Basque coast, although its presence is still evident in some sites and deserves further research.

In ***Chapter 2***, histopathology was used to assess the general health status of mussels (*Mytilus galloprovincialis*) collected from five sites contaminated with distinct patterns of organic and inorganic contaminants. Likewise, Chapter 2 presented the development of a detailed semi-quantitative histopathological index for the digestive gland and gonad of mussels. A total of twenty-three histopathological alterations were analysed in digestive gland and gonad following a weighted condition indices approach and were integrated into a single value for a better understanding of mussels' health status. According to the outcomes, mussels from the most impacted sites endured the most significant deleterious effects. In contrast, in moderate or low impacted sites, contamination levels did not cause significant tissue damage. However, parasites contributed to overestimating histopathological indices values (*i.e.* more severe tissue damage) in mussels from lowly impacted sites, whilst the opposite occurred in mussels from highly polluted sites. Accordingly, inter-site differences were more pronounced in autumn when natural physiological responses of advance maturation stages did not interfere in the histological response. Thus, if fluctuations of these confounding factors (seasonality and parasitosis) are carefully considered, digestive gland and gonad histopathological indices may provide good sensitivity for evaluating the general health status of mussels.

In Chapters 3 to 5, marine general health status was assessed through the use of different fish species. In ***Chapter 3***, multi-organ (liver, gills, kidney, spleen and gonad) histopathology in gobies (*Pomatoschistus* spp.), together with metal bioaccumulation and sediment contamination levels, were studied for the estuarine environmental risk assessment of the Ibaizabal estuary (SE Bay of Biscay). For that, four transects were investigated in a three year survey. Findings indicated that sediments were strongly-moderately impacted by metals and organic compounds, suggesting that adverse biological effects could be likely. Accordingly, metal bioaccumulation levels and multi-organ histopathological indices showed similar levels and affection degree in gobies collected along the estuary. However, in the last survey, both metal bioaccumulation levels and histopathological indices decreased, reflecting a lower impact on fish general health status. The liver, gills and kidney yielded higher histopathological damage than the spleen and gonad supporting their role in contaminant detoxification processes. Fat vacuolation of hepatocytes, lamellar fusion and melanomacrophage centers were the most prevalent hepatic, branchial and renal alterations, respectively. These histopathological changes may indicate exposure to non-specific toxicants, although the influence of other unknown environmental factors should not be excluded.

In ***Chapter 4***, common sole (*Solea solea*) and European hake (*Merluccius merluccius*), together with sediments, were collected during two campaigns along the

Basque continental shelf in an attempt to determine the use of fish histopathology under the scope of Descriptor 8 of the MSFD. For that purpose, the use of these potential sentinel fishes as well as the application of two existing histopathological indices (Costa et al. 2009 *versus* Lang et al. 2006) was compared and evaluated. According to the findings, metal bioaccumulation levels and histopathological lesions recorded in the liver were higher in common sole than in European hake which indicated that common sole due to its benthic behaviour was more sensitive as sentinel organism than European hake. On the other hand, the comparison of two existing histopathological indices revealed that both approaches were significantly correlated in both organs highlighting their suitability and accuracy. As for fish histopathology, no gross alterations classified as non-specific and early non-neoplastic toxicopathic lesions were observed in the liver and gonad of both species indicating slightly disturbed health status. These results were consistent with the sediments from the Basque continental shelf which were found to be moderately impacted by metals, but non-impacted by organic compounds. Furthermore, the lack of correlation between sediment contamination levels, metal bioaccumulation levels and histopathological indices suggested that other factors, rather than pollution alone, were responsible for the biological effects observed. Overall, fish histopathology is likely to be a suitable indicator of environmental pollution along the Basque continental shelf. However, for the implementation of histopathological assessment in sentinel organisms from offshore environments, the need to establish natural baseline levels and the influence of potential confounding factors is crucial.

Therefore, in ***Chapter 5*** hepatic and gonad histopathology, in combination with biometric parameters and hepatic metal bioaccumulation, was assessed monthly during one-year period in common soles from the Basque continental shelf, presumably a pristine area. Results indicated that Zn bioaccumulation levels presented a main peak in pre-spawning females, while Cd, Cu, and Pb demonstrated a seasonal bioaccumulation pattern in both genders, which mandates caution when interpreting these data without considering biological and environmental variations. Furthermore, seasonality and gender may act as confounding factors in biological indices and gonad histopathological index indicating again that sampling period and gender should not be neglected in sampling design and subsequent data interpretation. The most suitable period to assess these parameters is likely during the early-mid gametogenic stages, from June to September. However, although the prevalence of individual hepatic histopathological alterations showed seasonal changes, seasonality, gender and age may not be critical when addressing liver histopathological index in common soles, which might be beneficial for biomonitoring purposes.

Finally, in the ***General discussion***, the results obtained in the different Chapters are analysed from an integrative point of view, in relation to the objective established for this thesis. Overall, the results of the present work supported that an integrated assessment of environmental pollution combining biological effects in different sentinel organisms and sediment and/or tissue chemistry was an appropriate approach for evaluating the health status of marine environments. However, for a correct data interpretation the importance of setting the interference of confounding factors as well as the environmental assessment criteria is highlighted. Thus, these findings could contribute to the better assessment of environmental pollution under the premises of environmental policies such as Descriptor 8 in the MSFD.

General introduction

1. Pollution of aquatic environments

The aquatic environment is highly fragile, complex and diverse and involves different ecosystem types, such as rivers, lakes, estuaries, coastal areas and open oceans. These systems provide goods and services and support different uses that should be managed in a sustainable manner (Borja et al., 2008; Law et al., 2010; Lehtonen et al., 2014; Pascual et al., 2012). Although the aquatic systems have a tremendous capacity to assimilate human impact, it is currently known that we are overstressing our continental, estuarine, coastal and offshore waters. This anthropogenic impact on aquatic environments include physical and chemical transformation, habitat destruction and changes in biodiversity (Crain et al., 2009; Halpern et al., 2007; Kappel, 2011). The pollution of aquatic systems is one of the most important environmental problems that affects living aquatic life, goods and services provisions and human health via food web (Borja et al., 2010; Pascual et al., 2012; Schwarzenbach et al., 2006).

In general, aquatic systems present a variety of contaminant sources, such as atmospheric inputs (*e.g.* combustion), inland sources (*e.g.* agriculture, sewage, industry and urban run-off) and offshore activities (*e.g.* oil and gas extraction) (Figure I.1) (OSPAR, 2010). It is broadly accepted that all contaminants that enter into the ecosystem are due to anthropogenic activities (Halpern et al., 2007), although there is a background concentration for many chemicals, such as metals and metalloids (Cardoso et al., 2010; Fdez-Ortiz de Vallejuelo et al., 2010; Rodríguez et al., 2006; Tueros et al., 2008). This intensive anthropogenic input of contaminants may thereby cause marine pollution which, according to GESAMP (1991), is defined as "the direct or indirect introduction by man of substances or energy into the marine environment (including estuaries) resulting in deleterious effects, such as harm to living resources, hazards to human health, hindrance to marine activities including fishing, impairment of quality for use of sea water and reduction of amenities".

Figure I.1. Schematic overview of the main sources of hazardous substances and their pathways to aquatic environment (OSPAR, 2010).

The complexity of the environmental assessment of aquatic contamination is related to the wide range and type of sources (*i.e.* point and diffuse), the presence of different types and forms of chemicals and the varying scale of contamination (Meybeck and Helmer, 1996). This has led to the need to develop challenging integrative tools and methodologies to assess and manage aquatic health status (Borja et al., 2010; Izagirre et al., 2014; Martín-Díaz et al., 2008; Montero et al., 2013; Sousa, 2008). For this reason, nowadays intensive actions and strategies are being undertaken for the protection of aquatic systems and overall management of contaminant inputs (Borja et al., 2008; EEA, 2003; Ferreira et al., 2007; Lyons et al., 2010; Zampoukas et al., 2012).

2. Legislation for the management of aquatic systems in Europe

Over recent decades, the European Union (EU) has adopted water policies focusing on the integrative management of aquatic systems, based on the DPSIR approach (Driver-Pressure-State-Impact-Response, adopted by the European Environment Agency (EEA)) and the ecosystem-based approach (Atkins et al., 2011; Borja et al., 2006a, 2011; Hutchinson et al., 2013). These policies aim to restore and protect the ecological quality of aquatic systems, while ensuring the protection of marine waters, habitats and resources (Borja et al., 2008; Canessa et al., 2007; O'Boyle and Jamieson, 2006; Parson, 2005; Ricketts and Harrison, 2007). In Europe, four Regional Sea Conventions (RSCs); Barcelona Convention in the Mediterranean Sea (MEDPOL), Helsinki Convention in the Baltic Sea (HELCOM), Bucharest Convention in the Black Sea (BSC) and Oslo and Paris Convention in the North-Atlantic Ocean (OSPAR); provide suitable regional frameworks in order to implement international environmental laws (UNEP, 2007). Within the recent European legislation, the most relevant directives for the protection and restoration of the marine environment are (*1*) the Water Framework Directive (WFD, Directive 2000/60/EC), establishing a framework for Community actions in the field of water policy and (*2*) the Marine Strategy Framework Directive (MSFD, Directive 2008/56/EC) that promotes the protection of coastal and marine waters (Figure I.2). The main objective of both directives is to achieve and/or maintain a “Good Status” of marine waters, habitats and resources on the basis of an ecosystem-based approach (Borja et al., 2010). Both directives (see sections 2.1. and 2.2.) are of great relevance and are used as the basis to assess the health status of different aquatic environments (*i.e.* estuaries, coast and offshore waters) researched within this thesis.

2.1. The Water Framework Directive (WFD)

The WFD (2000/60/EC) is one of the most important pieces of environmental legislation produced in Europe in recent years and it establishes water policies for the protection and prevention of inland surface, transitional, coastal (until the first nautical mile (nm) from the coastal baseline) and groundwaters (Figure I.2) (Borja, 2005; Ferreira et al., 2007; Wernersson et al., 2015). The main objective of this directive is to achieve the “Good Ecological and Chemical Status” of all water bodies throughout Europe by 2015 and this is to be achieved by implementing management plans at the river basin level (Allan et al., 2006; Borja, 2005; Hering et al., 2010; Zabel et al., 2001).

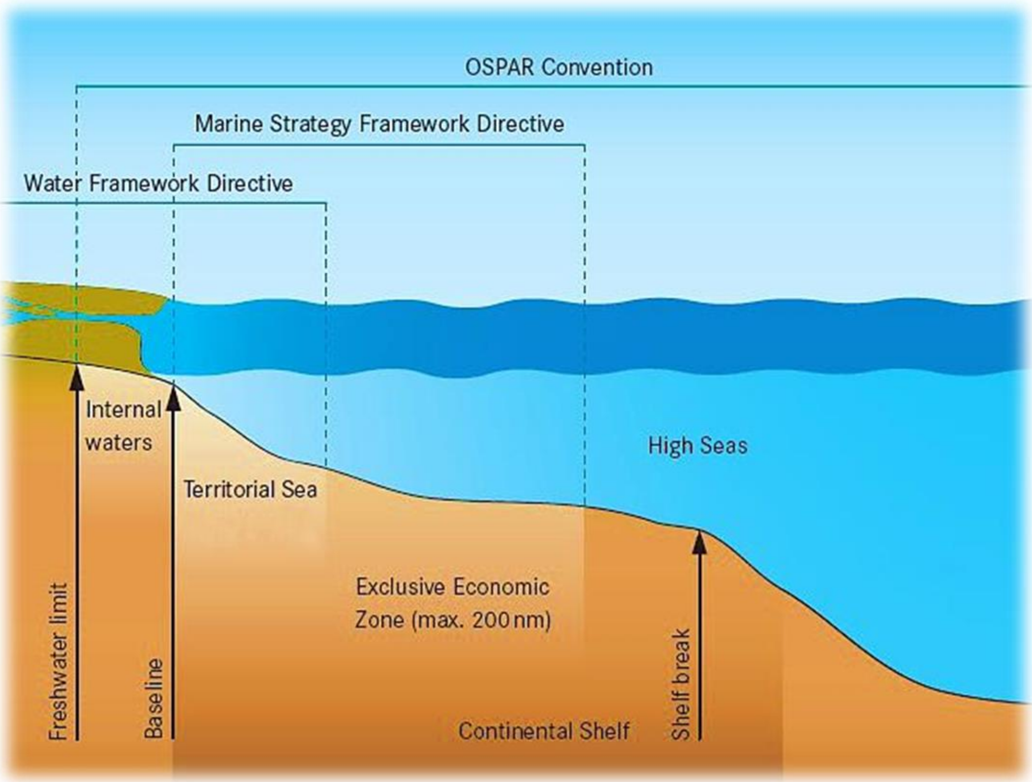

Figure I.2. Jurisdictional zones of the United Nation Conventions of the Law of the Sea, OSPAR Convention, WFD and MSFD (picture taken from http://qsr2010.ospar.org/en/ch01_02.html).

For this purpose, the WFD sets three types of monitoring depending on the objectives and type of information needed (Allan et al., 2006; Ferreira et al., 2007; Heiskanen et al., 2004; Rodríguez et al., 2010; Sanchez and Porcher, 2009):

(1) Surveillance monitoring: to provide information to assess long-term changes in natural conditions and changes resulting from widespread anthropogenic activities; to supplement and validate the impact assessment procedure; and to design efficient and effective upcoming monitoring programmes.

(2) Operational monitoring: to establish the status of water bodies identified as being at risk of failing to meet their environmental objectives; and to assess any changes in the status of such bodies resulting from the monitoring programmes.

(3) Investigative monitoring: to understand the reason for any unknown excessive ecological and chemical status; when surveillance monitoring indicates that the objectives for a water body are not likely to be achieved; or to determine the magnitude and impact of “accidental” pollution.

It is stated that Member States should establish surveillance and operational monitoring programmes as part of the river basin management plan, while in some cases the need to establish investigative monitoring programmes has been identified (Ferreira et al., 2007; Wernersson et al., 2015). In short, the final aim of these monitoring activities is to provide relevant information about the status of the different quality elements and priority substances in order to establish the “Ecological and Chemical Status” of water bodies (Annex VIII, 2000/60/EC). The quality elements (Figure I.3) that should be monitored within this policy in transitional and coastal waters are:

(1) Biological elements: composition, abundance and biomass of phytoplankton and composition and abundance of other aquatic flora, benthic invertebrate fauna and fish fauna (only in transitional waters).

(2) Hydromorphological elements: morphological conditions and tidal regime.

(3) Chemical and physico-chemical elements: general data such as transparency, thermal and oxygenation conditions, salinity and nutrient conditions, and synthetic and non-synthetic pollutants.

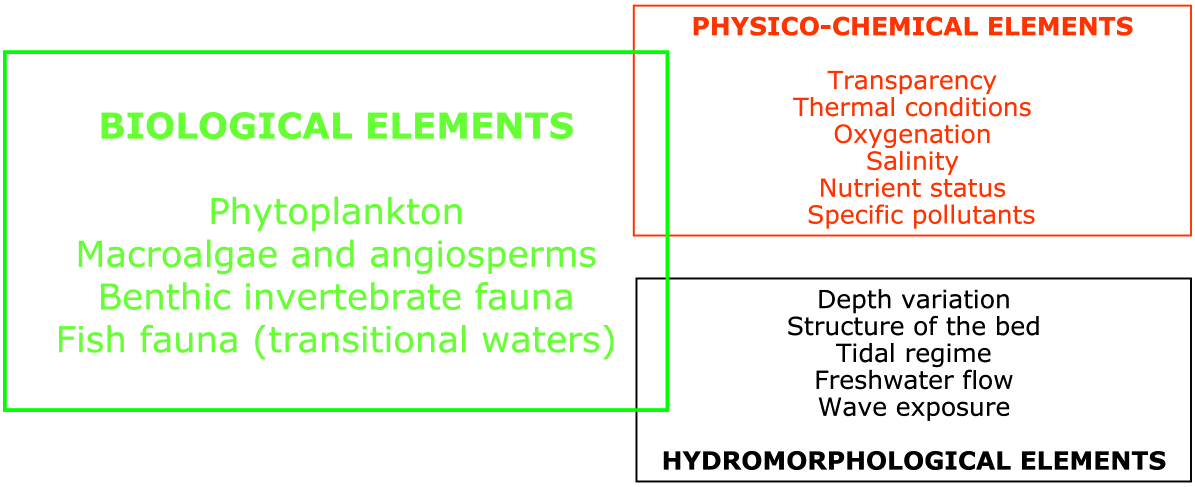

Figure I.3. Quality elements to be monitored for the assessment of the “Ecological Status”, according to the WFD.

The assessment of “Ecological Status” under WFD is based on the comparison between the quality elements in the water body to be assessed and those in undisturbed conditions (reference characteristics) for the same water types. These comparisons are quantified in Ecological Quality Ratios (EQRs) (Directive 2008/105/EC). On the other hand, the “Chemical Status” assessment relies on compliance with legally adopted Environmental Quality Standards (EQSs) for selected priority chemical pollutants of EU-wide concern that are set out in the Directive 2008/105/EC, recently amended by Directive 2013/39/EU. The WFD defines EQSs as “the concentration of a particular pollutant or group of pollutants in water, sediment or biota which should not be exceeded in order to protect human health and the environment" (Article II, 2000/60/EC). This implies that priority substance concentrations exceeding the EQS fail to achieve the chemical status (Menchaca et al., 2012; Strand, 2003; Tueros et al., 2009). The majority of these chemical substances are organic compounds, although four metals (*i.e.* Cd, Ni, Pb and Hg) are also considered as priority substances (Annex X, 2000/60/EC).

2.2. Marine Strategy Framework Directive (MSFD)

This directive (Directive 2008/56/EC), which aims to achieve and/or maintain "Good Environmental Status" (GES) in the marine environment by 2020 at the latest, covers all waters from the coastal baseline to 200 nm, which is known as Exclusive Economic Zone (EEZ). Therefore, WFD and MSFD spatially overlap in the coastal area as the WFD extends to 1 nm from the coastline (Borja et al., 2010) (Figure I.2). In the context of the MSFD, eleven qualitative descriptors are considered for determining GES (Table I.1).

Among the eleven descriptors, Descriptor 8 "Concentrations of contaminants are at levels not giving rise to pollution effects" is of great relevance for this thesis (Table I.1). This Descriptor indicates the importance of knowledge of contaminant levels in different matrices (*i.e.* water, sediment and biota) and their respective biological effects (Borja et al., 2011; Gago et al., 2014; Lyons et al., 2010; Tornero and Ribera d'Alcalà, 2014). In this attempt, most of the chemical compounds found within the marine environment are frequently those that are persistent, toxic and bio-accumulative, since it is not possible to analyse, detect and quantify all the substances present in the aquatic environment under MSFD premises (Gago et al., 2014; Hutchinson et al., 2013; Law et al., 2010). Chemical monitoring is therefore usually focused on already regulated chemical substances (priority substances as in WFD) that are known to cause a threat to the aquatic environment (Borja et al., 2011; Gago et al., 2014; Sanchez and Porcher, 2009).

Table I.1. Qualitative descriptors to be used in the assessment of the environmental status of marine waters in the context of the MSFD.

		Descriptors
1	*Biological diversity*	Biological diversity is maintained. The quality and occurrence of habitats and the distribution and abundance of species are in line with prevailing physiographic, geographic and climatic conditions
2	*Non-indigenous species*	Non-indigenous species introduced by human activities are at levels that do not adversely alter the ecosystems
3	*Exploited fish and shellfish*	Populations of all commercially exploited fish and shellfish are within safe biological limits, exhibiting a population age and size distribution that is indicative of a healthy stock
4	*Food webs*	All elements of the marine food webs, to the extent that they are known, occur at normal abundance and diversity and levels capable of ensuring the long-term abundance of the species and the retention of their full reproductive capacity
5	*Human-induced eutrophication*	Human-induced eutrophication is minimised, especially adverse effects thereof, such as losses in biodiversity, ecosystem degradation, harmful algae blooms and oxygen deficiency in bottom waters
6	*Seafloor integrity*	Sea-floor integrity is at a level that ensures that the structure and functions of the ecosystems are safeguarded and benthic ecosystems, in particular, are not adversely affected
7	*Hydrographical conditions*	Permanent alteration of hydrographical conditions does not adversely affect marine ecosystems
8	*Contaminants*	Concentrations of contaminants are at levels not giving rise to pollution effects
9	*Contaminants in fish and seafood*	Contaminants in fish and other seafood for human consumption do not exceed levels established by Community legislation or other relevant standards
10	*Litter*	Properties and quantities of marine litter do not cause harm to the coastal and marine environment
11	*Energy and noise*	Introduction of energy, including underwater noise, is at levels that do not adversely affect the marine environment

Nevertheless, only chemical determination does not provide enough toxicological information, being necessary the integrative assessment of biological effects-based measurements (Borja et al., 2010; Cardoso et al., 2010; Lyons et al., 2010). Besides, unlike in purely chemical monitoring, the determination of adverse biological effects provides enough toxicological information to estimate the risk caused at different levels of biological organisation by the large number of substances (mixture of contaminants) that are present in the aquatic environment, as well as to detect contaminants of emerging concern, metabolites and transformation products (Lyons et al., 2010; Wernersson et al., 2015).

Many of the monitoring methodologies applied in the WFD could be implemented within the MSFD, which may provide an easier and reliable way to implement this complex Directive (Borja et al., 2011; Zampoukas et al., 2012). For instance, the MSFD requires biological effect-based tools to be included in the assessment of marine environmental health status, while the WFD relies only on chemical tools to be involved in surveillance and operational monitoring programmes. However, biological effect-based tools, could be used for investigative monitoring to offer a valuable support in the assessment of water body quality (Borja et al., 2011; Ferreira et al., 2007; Hagger et al., 2009; Hering et al., 2010; Zampoukas et al., 2012).

3. Integrated assessment of environmental pollution

Aquatic organisms are exposed to a wide range of contaminants, which may cause diverse metabolic disorders, increase in disease frequencies and effects on community through alterations in factors such as growth, reproduction and survival (Borja et al., 2006b; Costa et al., 2011; Lang et al., 2006; Porte et al., 2006; Stentiford et al., 2014). Thus, the evaluation of potential adverse biological effects has been recommended as part of an integrated assessment of environmental pollution in field studies under WFD and MSFD premises (Borja et al., 2010; Gago et al., 2014; Hagger et al., 2008; Lyons et al., 2010).

In agreement, different authors concluded that a combination of suitable sets of chemical and biological measurements is the best way for monitoring and assessing aquatic health status (Borja et al., 2008; Chapman et al., 2013; Davies and Vethaak, 2012; Lyons et al., 2010). There are different ways to integrate biological and chemical data, such as the well-known “weight of evidence” (WOE) methodology. The WOE concept refers to the integration of findings recorded within a multidisciplinary approach, which comprises data from different lines of evidence (LOEs) like chemical, physico-chemical, ecotoxicological and biological data (Benedetti et al., 2014; Carreira et al., 2013; Martins et al., 2012; Morales-Caselles et al., 2008). These LOEs manage questions related to the presence of biological effects and chemical pollutants (Benedetti et al., 2014; Martín-Díaz et al., 2008). WOE methodologies are usually the basis of Ecological Risk Assessment (ERA), which agrees with the WFD and MSFD scopes (Benedetti et al., 2014; Leung et al., 2006; Martín-Díaz et al., 2008; Martins et al., 2012).

However, this thesis mainly follows the scheme of the integrated environmental pollution assessment of diverse ecosystem components proposed by the joint ICES/OSPAR Study Group on the Integrated Monitoring of Chemicals and their Effects (SGIMC) in cooperation with the ICES Working Group on Biological Effects of Contaminants (WGBEC) for European Regional Seas (Davies and Vethaak, 2012). This integrated environmental pollution assessment approach is based on three components: water, sediment and biota, which are evaluated by diverse methodologies (Figure I.4). Some of those methods are considered fundamental to the integrated assessment of environmental pollution and are described as “core methods”, while additional methodologies have been found to add value to the integrated assessment. These latter techniques have been described as “additional methods” and are not considered essential.

The integrated assessment approach pursued in this thesis is mainly focused on sediment (section 3.1.) and biota (section 3.2.) components. The evaluation of water component was discarded since water assessment is highly variable and unlikely to be representative of sampling sites, missing episodic pollution (Allan et al., 2006; Borja et al., 2004; Tueros et al., 2008). Furthermore, water assessment relies on compliance with already established EQSs (Directive 2013/39/EU; Strand, 2003; Tueros et al., 2009).

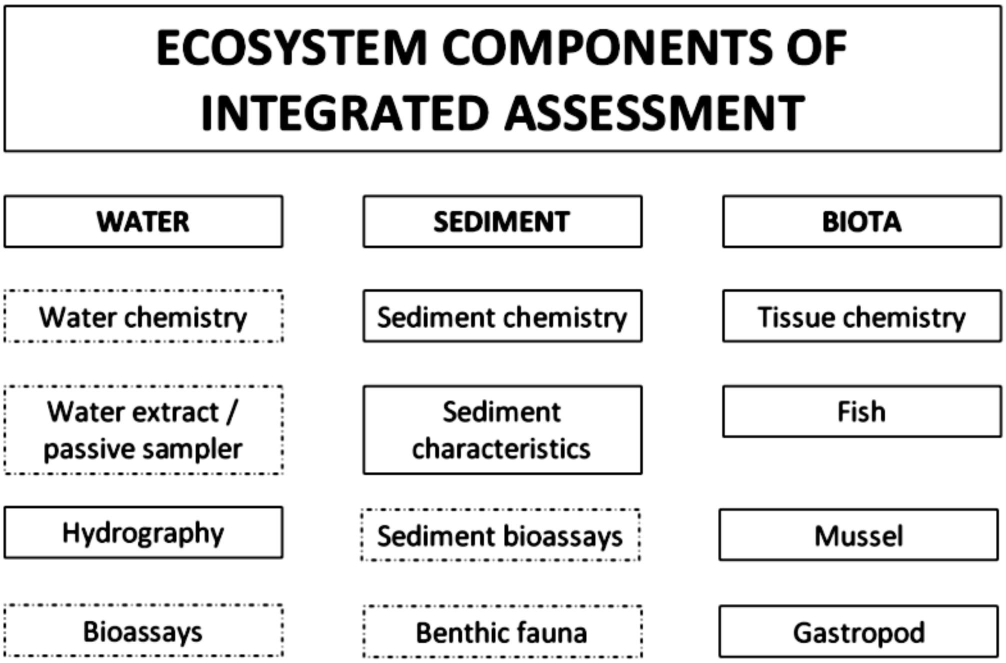

Figure I.4. Overview of ecosystem components for an integrated assessment of environmental pollution. Solid lines, core methods; broken lines, additional methods (Davies and Vethaak, 2012).

3.1. Sediments

Sediments are an essential, integral and dynamic part of the water column (Antizar-Ladislao, 2009; Belzunce et al., 2004). Thus, they have ecological, social and economic value due to their provision of habitats and food resources to benthic and demersal species (Apitz, 2012). However, sediments are a major environmental sink for many contaminants, since many chemicals originally introduced into the system by water (*e.g.* urban run-off) and sediment (*e.g.* tailing disposals and dredged materials) have affinities for sediment particles, which afterwards are deposited in estuaries and coastal systems (Martínez-Lladó et al., 2007).

Furthermore, sediments offer diverse advantages for chemical environmental assessment with respect to water: (*1*) physico-chemical properties of sediments (*e.g.* grain size, redox, particulate organic carbon and nitrogen), together with the geochemical characteristics of the environment (*e.g.* pH and salinity), determine the conditions leading sediments to behave as a sink of contaminants (Bocchetti et al., 2008; Fdez-Ortiz de Vallejuelo et al., 2014; Montero et al., 2013); (2*)* no inherent variability is found in sediments (Borja et al., 2004; Reczynski et al., 2010); (*3)* a quantifiable measurement of low concentration of chemical substances is feasible in sediments (Chapman et al., 2013; Tueros et al., 2008); *(4)* chemicals have a high affinity with sediment particles until their deposition allowing quantifiable measurements (Chapman et al., 2013) and *(5)* sediments constitute an appropriate matrix for the determination of the site-specific pollution events owing to their time-integrated information about the historical and current contaminant inputs (Reczynski et al., 2010; Rodríguez et al., 2006; Viguri et al., 2007).

In order to determine the contamination levels in sediments, different authors have established background concentration levels for those contaminants naturally present in the environment through different methodologies (Borja et al., 2008; Hinck et al., 2007; Zampoukas et al., 2012). In the case of synthetic substances, such as polychlorinated biphenyls (PCBs), there are no natural levels; thus background levels close to zero are considered (OSPAR, 2009). Sediment contamination levels are calculated by the ratio between the current level of a specific contaminant and the background value estimated for the corresponding area (Cearreta et al., 2000; Gredilla et al., 2013; Legorburu et al., 2013; Tornero and Ribera d'Alcalà, 2014), as well as by the estimation of indices such as the Geoaccumulation Index (Müller, 1979), Contamination Factor (Hakanson, 1980) and Enrichment Factor (Tomlinson et al., 1980).

Due to limited insight of ecological impact in these latter techniques, a more effective interpretation is recommended requiring tools that link contamination content of sediments with potential adverse biological effects (Montero et al., 2013; Oliva et al., 2013; Rojo-Nieto et al., 2014). Therefore, other methodologies, such as sediment quality guidelines (SQGs) combine chemical information with biological effects, providing a proxy to the potential toxicity of sediments (Borja et al., 2011; Costa et al., 2012; Long and MacDonald, 1998; Martín-Díaz et al., 2008). The SQGs express the relations between each potentially toxic chemical and the adverse effects it causes on organisms (Long and MacDonald, 1998). They are not definitive indications of whether harm will occur, although typically three levels are set (MacDonald et al., 1996): *(1)* concentrations below which adverse effects are unlikely (Threshold - Effects Level, TEL); *(2)* concentrations above which adverse effects are likely (Probable - Effects Level, PEL) and *(3)* intermediate concentrations within which adverse effects may or may not occur (> TEL < PEL). Nevertheless, important limitations of these methodologies have been also described (Burton, 2002; McCauley et al., 2000). For instance, according to Chapman et al. (1999), the SQG should be used in the region where they were developed, *i.e.* guidelines derived on site-specific data are able to better predict the toxicity of contaminants for each specific aquatic environment.

Sediment bioassays and benthic fauna (Figure I.4) have not been considered within this thesis, since the sediment assessment in this work has been based on the already determined sediment background, TEL and PEL values (Macdonald et al., 1996; Menchaca et al., 2012, 2014, Rodriguez et al., 2006) which implicitly reflect potential toxic effects.

3.2. Biota

Most organisms living in the aquatic environment are sensitive to any changes in their environment, whether natural (*e.g.* increased turbidity during an overflow) or anthropogenic (*e.g.* chemical contamination or decreased of dissolved oxygen due to sewage inputs) (Friedrich et al., 1996). The effects of chemical substances on biota are dependent on a number of factors, such as bioavailability, bioaccumulation, biomagnification, toxicity and the capability of the organism to metabolise chemical substances (OSPAR, 2010). In order to assess the biota component within the integrated assessment scheme, SGIMC of the

ICES/OSPAR has proposed tissue chemistry analysis and biological effects examinations in several sentinel organisms (*i.e.* gastropods, mussels and fishes) (Figure I.4).

The selection of a good candidate as sentinel organism is therefore a crucial issue in the assessment of biota component (Cajaraville et al., 2000; Lyons et al., 2010; Wu et al., 2005). A suitable sentinel species can be defined as any organism that can be used as an indicator of exposure to and toxicity of a xenobiotic, due to the species' sensitivity, position in a community, likelihood of exposure, geographic and ecological distribution or abundance, for assessing the impact on environmental health status (Bartell, 2006; Cajaraville et al., 2000; Oehlmann et al., 1996).

In accordance with the integrated assessment scheme (Figure I.4), three different sentinel organisms (*i.e.* gastropods, mussels and fish) are recommended and a set of different evaluation methodologies are proposed (Figures I.5, I.6 and I.7) for each sentinel organism. In this thesis, two gastropod species (netted dog whelks, *Nassarius reticulatus* and *Nassarius nitidus)* have been selected for imposex assessment and organotin tissue chemistry (Figure I.5), one bivalve mussel (*Mytilus galloprovincialis*) has been chosen for histopathology and metal tissue chemistry (Figure I.6) and three fish species (goby, *Pomatoschistus* spp.; European hake, *Merluccius merluccius* and common sole, *Solea solea*) have been considered for metal tissue chemistry, liver histopathology, intersex and externally visible diseases (Figure I.7). The selection of these methodologies is based on the specificity to determine organotin compound exposure in the case of imposex, and on the relevance to reflect the global health status in the case of histopathology. Tissue chemistry, however, was assessed in an attempt to relate biological effects with contaminant burden.

Figure I.5. Specific methods included in the gastropod component of the integrated monitoring framework (Davies and Vethaak, 2012).

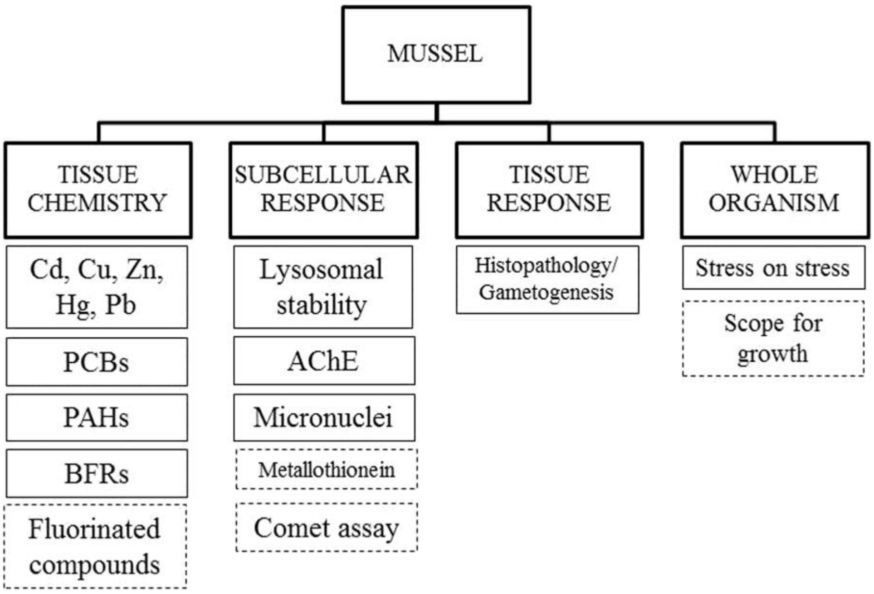

Figure I.6. Methods included in the mussel component of the integrated monitoring framework (Davies and Vethaak, 2012). Solid lines, core methods; broken lines, additional methods. PCBs, polychlorinated biphenyls; PAHs, polycyclic aromatic hydrocarbons; BFRs, brominated flame retardants; AChE, acetylcholinesterase.

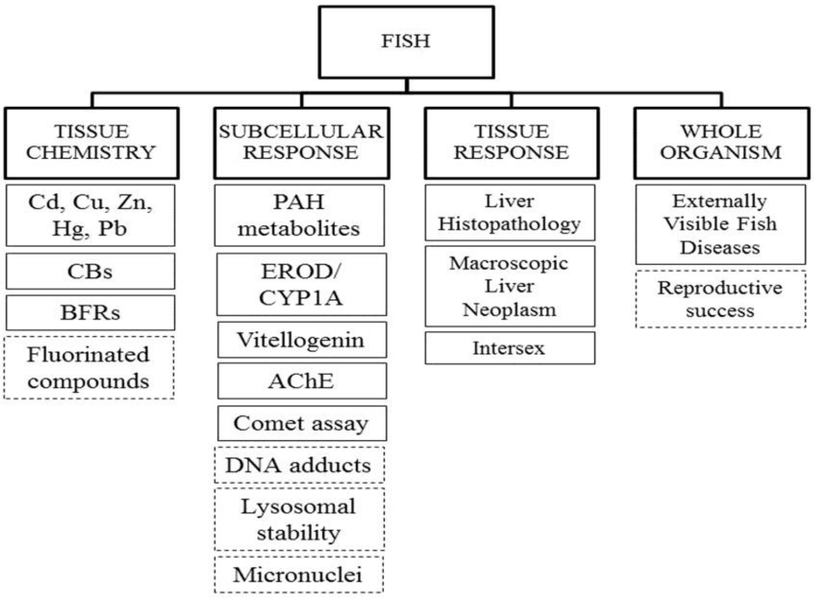

Figure I.7. Methods included in the fish component of the integrated monitoring framework (Davies and Vethaak, 2012). Solid lines, core methods; broken lines, additional methods. CBs, chlorinated biphenyls; BFRs, brominated flame retardants; PAH, polycyclic aromatic hydrocarbon; EROD: ethoxyresorufin-O-deethylase; CYP1A: cytochrome P4501A; AChE: acetylcholinesterase.

Several gastropod species have been widely employed as sentinel organisms to assess butyltin pollution (tributyltin (TBT) and its products) along the European Atlantic and the North Sea coasts (Barroso et al., 2005; Quintela et al., 2000; Rato et al., 2008; Rodríguez et al., 2009a, 2009b; Sousa et al., 2009). Within this thesis, two gastropod species (*N. reticulatus* and *N. nitidus*) were selected to evaluate organotin pollution along the Basque coast and estuaries (Figure I.8). *N. reticulatus* is found in offshore areas, such as open shores and bays (Santos et al., 2004) and is usually considered an intertidal species living down to a depth of 15 m on soft bottoms into which it burrows (Stroben et al., 1992). However, *N. nitidus* lives exclusively in bays and avoids wave-exposed areas along the Atlantic coast (Rodríguez et al., 2009a). Both gastropod species are thereby considered as complementary sentinel species concerning sampling surface coverage (Rodríguez et al., 2009a).

Figure I.8. General view of adult specimens of netted dog whelks used as sentinel organism within this thesis (*A*) *N. reticulatus* (Linaeus, 1758) and (*B*) *N. nitidus* (Jeffreys, 1867).

As for bivalve molluscs, mussels (*M. galloprovincialis*) have long been used as sentinel organisms to assess the health status of coastal and estuarine environments, owing to their tolerance to xenobiotic exposure and the respective wide range of biological responses (Figure I.9) (Besada et al., 2011; Marigómez et al., 2006; Shaw et al., 2011; Zorita et al., 2006). Besides, they are widespread within coastal and estuarine systems, sessile, filter-feeders and commercially important sea-food species (Gagnon et al., 2006). In comparison to other sentinel organisms, such as fish and crustaceans, bivalves have a very low metabolic activity, which reflects more precisely the magnitude of environmental contamination (Widdows and Donkin, 1990).

Figure I.9. Mussel (*M. galloprovincialis*) used as sentinel organism within this thesis (picture taken from http://l-aquaculture.blogspot.com.es/2012/12/mytilus-galloprovincialis.html).

In the case of teleosts, they have been targeted in ecotoxicological studies due to their ecological relevance, availability and ability to act as surrogates for higher vertebrates (Costa et al., 2009). The three sentinel fish species used in this thesis were selected to cover estuarine (gobies), coastal and offshore waters (common sole and European hake) (Figure I.10). Goby (*Pomatoschistus* spp.) is an ubiquitous and abundant species in most European estuaries (Martinho et al., 2006). These species spend their entire life cycle within estuaries in contact with sediments, being frequently impacted by hazardous substances (Dolbeth et al., 2007). Owing to their limited migrational tendencies, they are susceptible to showing pollution-induced lesions of a particular area (Arruda et al., 1993) and as such, the use of gobies as sentinel organisms in ecotoxicological studies has been demonstrated (Fonseca et al., 2013; Nyitrai et al., 2013; Stentiford et al., 2003). In offshore and coastal waters, flatfishes have been widely employed as sentinel species, in part owing to their benthic-like behaviour, which makes them appropriate sentinels for biomonitoring marine sediments (Stentiford et al., 2003). One of the most abundant flatfish of the Basque continental shelf is the common sole (*S. solea*) (Franco et al., 2012; Quincoces et al., 2011), which is characterised by a high habitat fidelity (Pawson, 1995). Similarly, the European hake (*M. merluccius*), although to a lesser extent than soles, has also been targeted as a sentinel organism within offshore and coastal environments (Bodiguel et al., 2009; Marigómez et al., 2006; Raingeard et al., 2009). Furthermore, European hake is both commercially and ecologically one of the most important demersal fish species in the Bay of Biscay (Sanchez and Gil, 2000), whereby this area is the biggest nursery of this species (Casey and Pereiro, 1995).

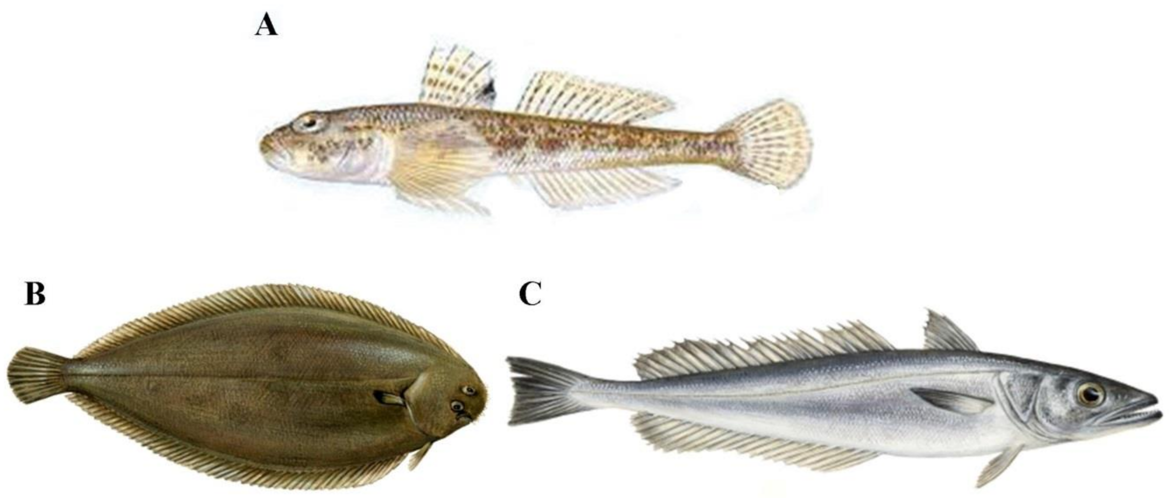

Figure I.10. Fish species used as sentinel organisms within this thesis: (*A*) goby (*Pomatoschistus* spp.), (*B*) common sole (*S. solea*) and (*C*) European hake (*M. merluccius*) (pictures taken from http://ec.europa.eu/fisheries/marine_species/index_en.htm).

3.2.1. Tissue chemistry

Persistent chemicals may accumulate in aquatic organisms through different pathways: via the direct uptake from water by gills or skin (bioconcentration), via uptake of suspended particles (ingestion) and via the consumption of contaminated food (biomagnification) (van der Oost et al., 2003). Thereby, tissue chemistry in biota measures the potential bioavailability of contaminants, which is defined as "the fraction of the bulk amount of a chemical present in water, sediment and interstitial water that can potentially be released into the organism's tissues" (Belfroid et al., 1996).

The characteristics of a chemical substance, such as its molecular size and polarity determine the association of the chemical with particles affecting its bioavailability (van der Oost et al., 2003). For instance, non-polar chemicals, such as PCBs, have low aqueous solubilities and a strong tendency to be associated with dissolved and particulate organic matter, which make them less bioavailable to non-sediment-ingestors. Ionic substances, such as certain metals, however, have high aqueous solubility and tend to be more bioavailable within the aquatic environment (EPA, 2000). Additionally, hydrophobicity of a contaminant, especially for organic substances, is one of the most important chemical characteristics determining the bioaccumulation behaviour of chemical compounds in aquatic systems (EPA, 2000).

On the other hand, bioaccumulation is a function of the bioavailability of contaminants in combination with species-specific uptake and elimination processes (EPA, 2000). For instance, some organisms may accumulate contaminants throughout their whole life time without detectable adverse effects on their normal physiological functions. These species may have detoxifying mechanisms which bind the contaminant in certain sites in the body and render them harmless (Annabi et al., 2013; Coelho et al., 2010; Friedrich et al., 1996). Other organisms accumulate contaminants over a period of time and only suffer adverse effects when critical levels are reached in their body tissues, or a change in their metabolic pattern re-releases some specific contaminants within their body (Friedrich et al., 1996; Teles et al., 2007; Zorita et al., 2006). Nevertheless, there are some organisms which are able to biotransform specific contaminants to more hydrophilic products, becoming non-bioaccumulative contaminants (*e.g.* PAHs in fishes) (van der Oost et al., 2003).

As already mentioned, chemical characterisation by itself does not provide specific biological information about potential hazards to aquatic organisms; but chemical data in biota from several trophic levels provide information on persistence, mobility, bioavailability, accumulation within food web and then risks related to human consumption (Franke et al., 1994; Gago et al., 2014; Jahnke et al., 2014; Magalhães et al., 2007; Monperrus et al., 2005). Besides, organisms collected from their natural habitat also provide a time-integrated measure of environmental concentrations of a contaminant, averaging out temporal fluctuations (Goldberg et al., 1986; Solaun et al., 2013; Sousa et al., 2009). Monitoring of contaminants in body burden of aquatic organisms is undertaken for the following purposes: *(1)* to assess the effectiveness of measures taken for the reduction of marine contamination (temporal biomonitoring) (*e.g.* Solaun et al., 2013; Sousa et al., 2009; Szlinder-Richert et al., 2008); *(2)* to evaluate the existing level of marine contamination within a study area (spatial distribution monitoring) (*e.g.* Cortazar et al., 2008; Fattorini et al., 2008) and *(3)* to assess the causes of adverse biological effects in living resources and marine life (*e.g.* Bodin et al., 2004; Giarratano et al., 2011; Triebskorn et al., 2008). However, the complexity of bioaccumulation should be considered including toxicokinetics, metabolism, organ-specific bioaccumulation and bound residues, to relate critical body burden concentrations with ecotoxicological endpoints (van der Oost et al., 2003).

In this respect, it is important to point out that contaminants tend to concentrate in specific organs in aquatic organisms (Monroy et al., 2014; Nesto et al., 2007; Paulino et al., 2014). For instance, persistent organic substances (*e.g.* POPs), which are highly lipophilic, accumulate mainly in fatty tissues, such as liver or digestive gland (Nanton, 2003). Thus, due to organ-specific bioaccumulation behaviour, recent studies have reported that the most promising bioaccumulation tissues for chemical exposure assessment are the main detoxification organs, *i.e.* liver/digestive gland, or muscle (due to their relation with human consumption), or whole body (especially in small individuals) (Coelho et al., 2010; Moore and Allen, 2002; Oliveira Ribeiro et al., 2005; Paulino et al., 2014; Ruiz et al., 2005). Therefore, in this thesis, liver was selected as a target organ in common sole and European hake and the whole organism in gastropods, mussels and gobies for tissue chemistry determination.

3.2.2. Biological effects

As mentioned above (see section 3), the assessment of environmental pollution should not be exclusively based on chemical analyses since this monitoring approach does not provide any indication of possible deleterious effects of contaminants on biota (Allan et al., 2006; Borja et al., 2011; Chapman et al., 2013; Lyons et al., 2010). One of the advantages of using biological effect techniques is that they indicate links between contaminant exposure and ecological endpoints, as well as detecting the impact of pollutants that may not be determined routinely within purely chemical monitoring approaches (Cajaraville et al., 2000; van der Oost et al., 2003; Viarengo et al., 2007).

In order to analyse the extent of disturbances of a biological system and to quantify its health status, the integration of the measurement of several biological effects at different levels of biological organisation has been suggested (Allen and Moore, 2004; Broeg et al., 2005; Cajaraville et al., 2000; Viarengo et al., 2007). In general, biological measurements are classified into three conceptual and methodological approaches (ICES, 2006): (*1*) biomarkers: at sub-organism level (*i.e.* molecular, cell and tissue level); (*2*) bioassays: at whole organism level and (*3*) ecological survey: at population and community level. As this work deals mostly with biomarkers we will adopt the definition proposed by McCarthy and Shugart, (1990). Hence, biomarkers are measurements of body fluids, cells or tissues at cellular, biochemical and molecular levels that indicate the presence of pollutants (exposure biomarkers) or the magnitude of the organism response (effects biomarkers). Owing to the short time required between the exposure to a chemical and the response of some biological endpoints, biomarkers are used as early warning signals to predict changes at higher levels of organisation, *i.e.* population, community or ecosystem (Allen and Moore, 2004; Bartell, 2006; Cajaraville et al., 2000; Chapman et al., 2013; van der Oost et al., 2003; Viarengo et al., 2007), which is of major importance because damage at the population and ecosystem level can take a long time to repair (Chapman et al., 2013; Hagger et al., 2008).

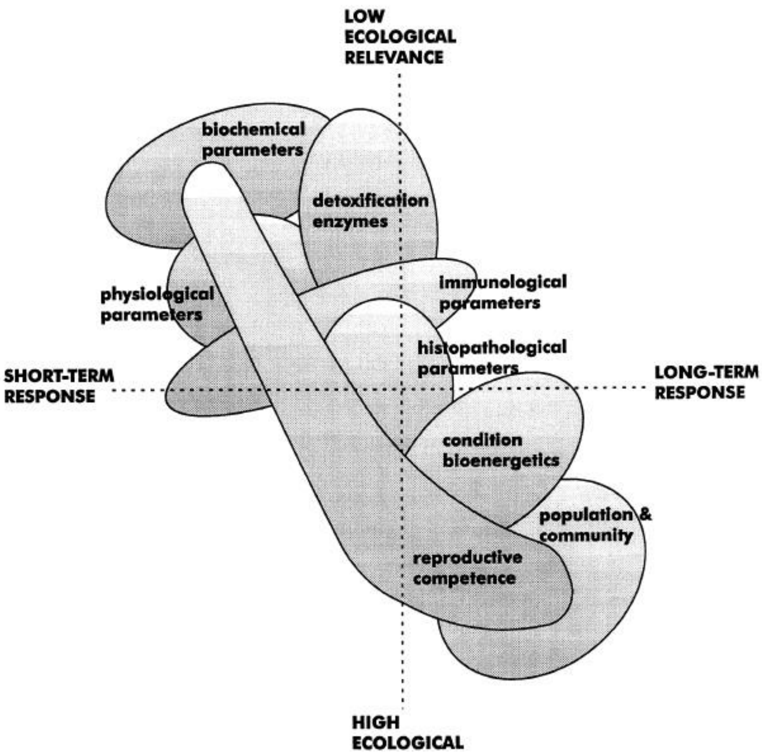

Figure I.11. Relationships between ecological relevance and time-scales of pollutant-induced biomarker responses (picture adapted from Adams et al. (1989)).

In general, biological responses at lower biological organisation level (biomarkers or bioassays) are more specific, sensitive, reproducible and easier to determine, but more difficult to relate to ecological alterations (Figure I.11). On the other hand, biological responses at more complex biological organisation level (ecological survey) are directly indicative of ecosystem health status, but rather more difficult to determine, less specific and only manifest at a late stage when environmental damage has already occurred (Bartell, 2006; Connell et al., 1999; van der Oost et al., 2003) (Figure I.11). Various research works proposed that a multi-biomarker approach, based on a combination of several types of biomarkers, is a useful tool to assess the impact of environmental pollution at different levels of biological organisation (Al-Subiai et al., 2011; Brenner et al., 2014; Broeg et al., 2005; Fonseca et al., 2011; Parolini et al., 2010; Santos et al., 2010; Viarengo et al., 2007; Zorita et al., 2007).

The relationship between a biomarker response and chemical exposure is not necessarily linear due to adaptive mechanisms (de Lafontaine et al., 2000; Mayer et al. 1992), antagonistic or synergistic conditions (Ahmad et al., 2005) or transient responses (Sanchez et al., 2005; Shaw et al., 2011). The ability of any biological method to indicate the state of the environment is dependent on the degree and duration of exposure to the pollutants, as well as the sensitivity and response rate of biological processes. Indeed, biomarkers can also respond to other factors of environmental stress (Bocchetti and Regoli, 2006; Cancio et al., 1999; Garmendia et al., 2010; Izagirre et al., 2014; Moore, 1991).

For that reason, one of the main disadvantages of using biological effect-based measurements in wild organisms for marine health status assessment is the variety of potential confounding factors that may hamper the evaluation of biological responses to pollutants (Cancio et al., 1999; Gonçalves et al., 2013; Leung et al., 2001; Lyons et al., 2010; Minguez et al., 2012). Therefore, the influence of potential confounding factors has to be regarded carefully in the design of the sampling strategy and the interpretation of biological data (Bignell et al., 2008; Brenner et al., 2014; Izagirre et al., 2014; Leiniö and Lehtonen, 2005; Vidal-Liñán and Bellas, 2013). In this attempt, a set of supporting information (*e.g.* gender, reproductive stage or age) should be simultaneously registered in order to reach an accurate and reliable assessment criteria (ICES, 2011). Furthermore, baseline levels should also be well established to successfully differentiate the pollution-induced effects from normal physiological and biological responses (Bebianno and Serafim, 2003; Brenner et al., 2014; Fricke et al., 2012; Schmidt et al., 2013).

As stated before, for the integrated assessment of environmental pollution (Figure I.4) we will focus on imposex and histopathological methods and therefore these will be treated in depth in the next sections (3.2.2.1 and 3.2.2.2).

3.2.2.1. Imposex

The adverse effects of organotin compounds are shown in a wide range of organisms, reflecting sub-lethal and lethal damage in microorganisms, invertebrates and vertebrates (Austen and McEvoy, 1997; Cruz et al., 2007; Martínez-Lladó et al., 2007). This biological damage includes acute toxicity, RNA alterations, neurotoxicity, teratogenicity and immunotoxicity. Therefore, TBT is classified as one of the most toxic xenobiotics that is introduced anthropologically to marine and estuarine waters (Goldberg, 1986; Ruiz et al., 2010). Besides, organotin compounds can be active at very low concentrations, producing adverse effects in aquatic organisms (Oehlmann et al., 2007; Oetken et al., 2004). In particular, this compound acts as an endocrine disruptor in many gastropod and bivalve species. For instance, several research studies have demonstrated that TBT can modify the morphology of sexual characters in dioic neogastropoda species, producing a phenomenon known as imposex, *i.e.* the imposition of male sexual characters onto female gastropods (Figure I.12). Thus, due to the correlation between TBT and imposex levels in some gastropod species, imposex has been proposed by international organisations, such as ICES and OSPAR, to assess the effects of organotin compounds in the marine and estuarine environments (ICES, 2000; OSPAR, 2008), being one of the few biomarkers that are mandatory within the OSPAR (OSPAR, 2008). Accordingly, many countries accomplish routine controls of imposex levels, such as the United Kingdom, the United States or France (Michel et al., 2001; O'Connor, 1996; Thomas et al., 2002); while other countries have carried out specific research work (Barroso et al., 2005; Pavoni et al., 2007; Rodríguez et al., 2009a, 2009b; Sousa et al., 2009). Additionally, the assessment of imposex in gastropod species has been reported as an effective biomonitoring method for assessing organotin compounds, as a cheaper and more sensitive methodology than measuring TBT chemical elements (Afsar, 2015; Sonak et al., 2009).

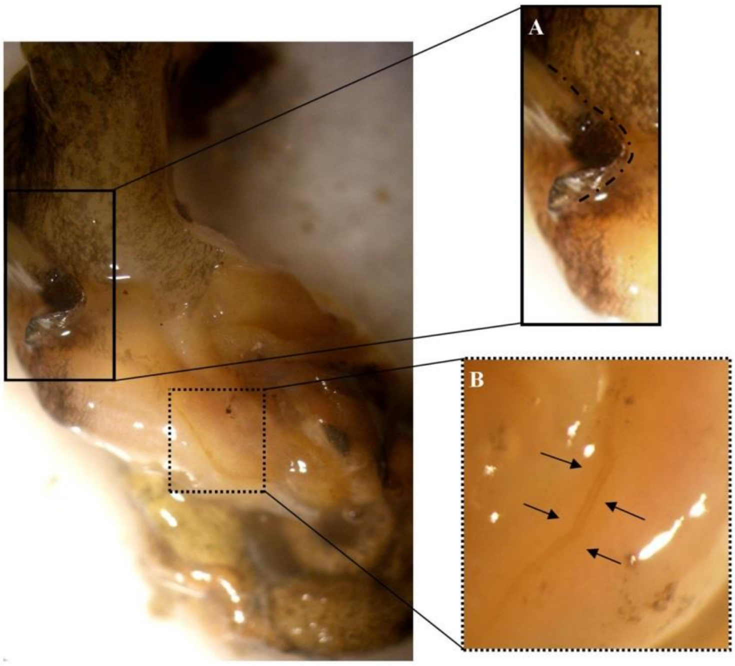

Figure I.12. Imposex phenomenon in a *N. reticulatus* female showing the presence of (*A*) a penis and (*B*) vas deferens duct.

3.2.2.2. Histopathology

Histopathology is defined as the study of diseases and dysfunction of natural biological processes at tissue and cell level. As such, histopathology is a valuable tool for assessing the health of individuals and populations, because it incorporates measures of reproductive and metabolic condition and allows the detection of a range of pathogens that may provoke morbidity and mortality (Bignell et al., 2008; Costa et al., 2013; Stentiford et al., 2003).

Moreover, measuring histopathological alterations in wild organisms has been considered one of the most important approaches to assess pollution-driven adverse effects to organisms in biomonitoring biological effects studies (ICES, 2009; Fricke et al., 2012; Stentiford et al., 2009; van Dyk et al., 2007; Vethaak and Jol, 1996). Histological effects are fundamentally biochemical (malfunctions in cellular metabolic processes) and, occasionally, morpho-physiological changes that become apparent at more complex levels of biological organisation (Morley, 2010; Sindermann, 1993). Therefore, it is well-known that pathological alterations are a reflection of disturbances at a simple level of biological complexity (Lang et al., 2006; Moore and Simpson, 1992). It provides an effective set of tools for the detection and characterisation of toxicopathic pathologies, which are increasingly being used as indicators of environmental stress in diverse target organs (Bignell et al., 2011; ICES, 2004) (*e.g.* Figure I.13). As an example, Tables I.2, I.3 and I.4 summarise the histopathological alterations commonly recorded in the digestive gland of mussels and in the liver of fishes.

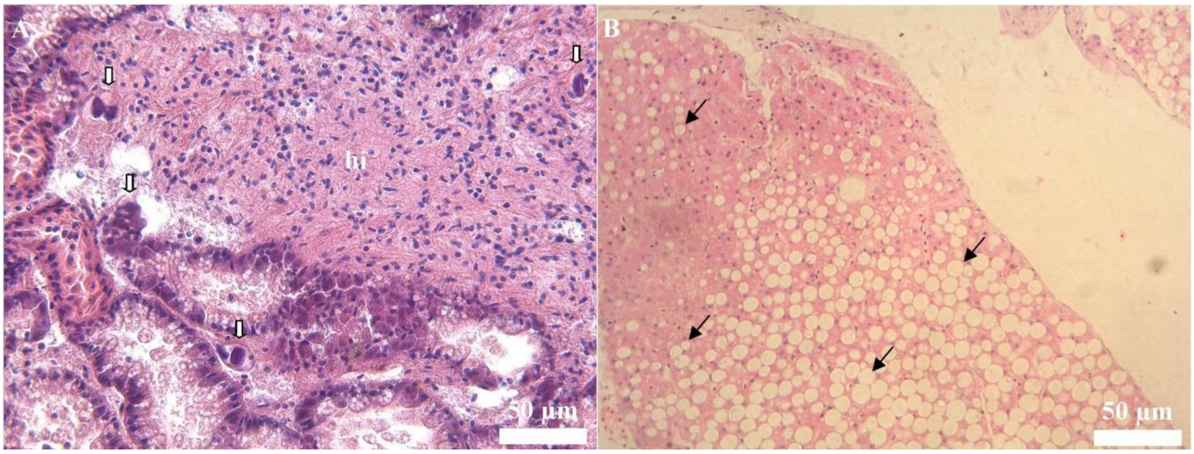

Figure I.13. Histopathological sections stained with H&E. (*A*) Haemocytic infiltration (hi) and presence of *Nematopsis* parasites (white arrows) in the digestive gland of a mussel collected in Mundaka and (*B*) fat vacuolation of hepatocytes (arrows) in the liver of a goby collected in the Ibaizabal estuary.

Table I.2. Summary of histopathological alterations commonly recorded in the digestive gland of mussels, together with their histological description, causal agent and potential confounding factors.

Alteration	Description	Causal agent	Confounding factor	References
Tubule atrophy	Thinning of the digestive tubule epithelium	Long-term exposure to pollutants (metals, pesticides, PAHs and mixture of xenobiotics)	Natural biotic and abiotic factors	Cajaraville et al., 1990 Da Ros et al., 1995 Marigómez et al., 2006 Garmendia et al., 2010
Haemocytic infiltration	Infiltration of haemocytes into connective tissue	Exposure to pollutants (metals, pesticides, PAHs and mixture of xenobiotics)	Natural biotic and abiotic factors Shell damage Infections	Da Silva et al., 2011 De los Ríos et al., 2012 Cappello et al., 2013 Izagirre et al., 2014
Granulocytomas	Clusters of haemocytes or the disintegration and deterioration of tissue	Long-term exposure to pollutants (metals, pesticides, PAHs and mixture of xenobiotics)	Natural biotic and abiotic factors Infections	Carballal et al., 1998 Bignell et al., 2008 Svärdh, 2011 Izagirre et al., 2014
Necrosis	Morphology of a cell or a tissue after irrevocable loss of cell function, presenting eosinophilic and pale staining with nuclear condensation.	Long-term exposure to pollutants (metals, pesticides, PAHs and mixture of xenobiotics)	Natural biotic and abiotic factors	Cajaraville et al., 1990 Al-Subiai et al., 2011 Vasanthi et al., 2012 Sheir et al., 2013
Brown cells	Pigmented macrophages	Exposure to pollutants (metals, pesticides, PAHs and mixture of xenobiotics)	Natural biotic and abiotic factors Infections	Zorita et al., 2006 Kim et al., 2008 Garmendia et al., 2010 De los Ríos et al,, 2013
Haemocytic neoplasia	Presence of single anaplastic cells with enlarged nuclei and sometimes frequent mitosis	Long-term exposure to pollutants (pesticides, PAHs and mixture of xenobiotics)	Natural biotic and abiotic factors	Alonso et al., 2001 Villalba et al., 2001 Ciocan and Inke, 2005 Zorita et al., 2007

Table I.3. Summary of histopathological alterations recorded in the liver of fish species, together with their histological description, causal agent and potential confounding factors.

Alteration	Description	Causal agent	Confounding factor	References
Melanomacrophage centres	Single or multiple pigmented (melanin) cells	Mixture of xenobiotics	Age Nutritional condition Reproductive stage Infections	Vethaak and Wester, 1996 Agius and Roberts, 2003 Stentiford et al., 2003 van Dyk et al., 2012
Haemorrhage	Blood leaking from vessels	Mixture of xenobiotics	Natural biotic and abiotic factors Infections	Loganathan et al., 2006 Geeraerts and Belpaire, 2010 Costa et al., 2011 Gonçalves et al., 2013
Hyperaemia	Congestion of blood vessels	Mixture of xenobiotics Organic compounds	Natural biotic and abiotic factors Infections	Noreña-Barroso et al., 2004 Agamy, 2012 Costa et al., 2011 Gonçalves et al., 2013
Haemosiderosis	Accumulation of haemosiderin deposits in hepatocytes	Mixture of xenobiotics PAHs and PCBs Petroleum	Natural biotic and abiotic factors Infections	Khan and Nag, 1993 Thiyagarajah et al., 1998 Khan, 2003 Fricke et al., 2012
Concentric periductal fibrosis of bile ducts	Proliferation of connective tissue surrounding bile ducts	Mixture of xenobiotics Metals	Natural biotic and abiotic factors Infections	Koheler, 2004 Della Torre et al., 2010 Lukin et al., 2011 Fricke et al., 2012
Lymphocytic infiltration	Infiltration of single or multiple lymphocytes aggregations	Mixture of xenobiotics	Natural biotic and abiotic factors Infections	Triebskorn et al., 2008 Lukin et al., 2011 Fricke et al., 2012 Agamy, 2012
Necrosis	Pathologic cell death showing nuclear chromatin condensation, pale staining and permeable cell membrane	Mixture of xenobiotics	Natural biotic and abiotic factors	Kent et al., 1988 Pacheco and Santos, 2002 Costa et al., 2011 Fricke et al., 2012

Table I.4. Continuation of the summary of histopathological alterations recorded in the liver of fish species, together with their histological description, causal agent and potential confounding factors.

Alteration	Description	Causal agent	Confounding factor	References
Granulomatosis	Numerous swelling formed of granulation tissue.	Mixture of xenobiotics	Natural biotic and abiotic factors Infections	Lang et al., 2006 Della Torre et al., 2010 Fricke et al., 2012 van Dyk et al., 2012
Eosinophilic bodies in hepatocytes	Cytoplasmic, well-defined and reddish inclusions	Mixture of xenobiotics Organic compounds Metals	Natural biotic and abiotic factors	Camargo and Martinez, 2007 van Dyk et al., 2007 Costa et al., 2009 Costa et al., 2011
Fat vacuolation of hepatocytes	Retention of lipids in vacuoles within the hepatocytes	Mixture of xenobiotics	Nutritional condition Reproductive stage	van Dyk et al., 2007 Giari et al., 2007 Costa et al., 2009 Domínguez-Petit et al., 2010
Hepatocellular and nuclear pleomorphism	Cells displaying marked abnormalities in cell or nuclear size	PAHs and PCBs Carcinogenic compounds	Natural biotic and abiotic factors Age	Myers et al., 1991 Stehr et al., 1998 Stentiford et al., 2003 Lang et al., 2006
Hydropic vacuolation of epithelial cells of bile ducts	Retention of fluids in vacuoles within epithelial cells of bile duct	Mixture of xenobiotics PAHs	Natural biotic and abiotic factors Age	Chang et al., 1998 Myers et al., 1991 Stehr et al., 1998 Lang et al., 2006
Spongiosis hepatis	Clusters of small cyst-like structures	Mixture of xenobiotics	Natural biotic and abiotic factors Age Reproductive cycle	Couch, 1991 Boorman et al., 1997 Ding et al., 2010 Agamy, 2012
Hepatocellular adenoma	A benign tumour or neoplasm composed of glandular tissue	Carcinogenic compounds	Natural biotic and abiotic factors Age	Vethaak and Jol, 1996 Reynolds et al., 2003 Stentiford et al., 2003 Köhler and Ellesat, 2008
Hepatocellular carcinoma	A malignant tumour whose parenchyma is composed by anaplastic epithelial cells	Carcinogenic compounds	Natural biotic and abiotic factors Age	Myers et al., 1991 ICES, 2004 Köhler and Ellesat, 2008 Lerebours et al., 2013

In histopathological studies, the selection of a suitable target organ is an essential issue that should be addressed (Gagnon et al., 2006; ICES, 2006; Soto et al., 1997). One of the most evaluated target organs in molluscs and fishes are the digestive gland and liver, due to aforementioned biological functions (see section 3.2.1.) (Bignell et al., 2011; Brenner et al., 2014; Cappello et al., 2013; Costa et al., 2009, 2013; de los Ríos et al., 2013; Izagirre et al., 2008; Lang et al., 2006; Moore and Allen, 2002; Stentiford et al., 2003). Gonads are also used as a target organ due to their fundamental function in hormone production, gametes development and progress of upcoming generations (Fernández-Reiriz et al., 2007). Furthermore, the gonad development analysis and gonad histopathology are fundamental supporting data included in the Mussel Watch Project (Hillman, 1993) because gonads play an essential role in environmental and physiological assessment of aquatic populations (Medina et al., 2012; Puy-Azurmendi et al., 2010; Thain et al., 2008). However, gonad histology and especially intersex condition is used to detect the effect of endocrine disrupting chemicals in the aquatic environment (Bizarro et al., 2013; Dias et al., 2014; Ortiz-Zarragoitia and Cajaraville, 2010; Teles et al., 2007).

In the integrated assessment of environmental pollution, histopathology is also complementary to other techniques used to monitor the biological effects of contaminants as it can help to dissociate markers of underlying health or disease condition from those associated with exposure to contaminants (Pacheco and Santos, 2002).

Among the different methodologies of histopathological quantification, the calculation of the prevalence of alterations has been widely used within biomonitoring studies. Prevalence detects the frequency of a histopathological lesion within a population (Fricke et al., 2012; Puy-Azurmendi et al., 2010; Sousa et al., 2009: Stehr et al., 1998). Nonetheless, the absence of some sort of quantification makes it difficult to statistically establish significant cause-effect relationships between pathology and contaminants and to assess significant differences between sampling areas or campaigns (Costa et al., 2009; Lang et al., 2006; van Dyk et al., 2012). Therefore, semi-quantitative histopathological approaches, especially those that consider the relative biological importance and dissemination degree of lesions, address this issue providing more sensitive findings of histopathological data (Costa et al., 2009; Lang et al., 2006, Lukin et al., 2011). Similarly, quantitative image-based assessment of histological changes has been also employed within biomonitoring biological effects in order to obtain more reliable and sensitive outcomes (Cajaraville et al., 1990; Garmendia et al., 2010; Múgica et al., 2015).

However, in order to obtain comparable data it is crucial to define or standardise diagnosis criteria since this is the most subjective aspect of the approach and depends on the expertise of the pathologist. Hence, to guarantee data of quality different methods have been proposed. One of these methods consists of a blind check of 10 – 15% of previously diagnosed samples by the same or another pathologist (Zorita and Cuevas, 2014). And the other method is based on the participation in intercalibration exercises and training workshops. For instance, there are quality assurance programmes, such as Biological Effects Quality Assurance in Monitoring Programmes (BELQUAM) that organise intercalibration exercises and training workshops in order to define quality standards (http://www.bequalm.org/eu_project.htm). On the other hand, comparable histopathological data is also obtained when different existing indices that follow different classification systems are able to give similar results. Thus, it is of paramount importance to compare and contrast different histopathological indices to validate their use.

4. The Basque coast

The study area of this thesis, the Basque Coast, is situated in the SE Bay of Biscay (Figure I.14), bordering with France in its eastern limit and with Cantabria Region in its western limit. The length of the Basque coast is approximately 150 km, while the width of its continental shelf is characterised by its narrowness, ranging between 7 and 20 km (Uriarte et al., 1998). In this territory, coastal waters are highly influenced by terrestrial climatic conditions and the entrance of open ocean water masses (Valencia et al., 2004). The Basque littoral is mostly composed of cliffs of calcareous rocks with small beaches and many short rivers flowing to the shelf, mainly through estuaries (Cearreta et al., 2004). These estuaries, in general, are relatively small (< 25 km), shallow (5 – 10 m), with low residence time (< 1.5 days) and are heavily influenced by the river and the sea tide (Borja et al., 2006a).

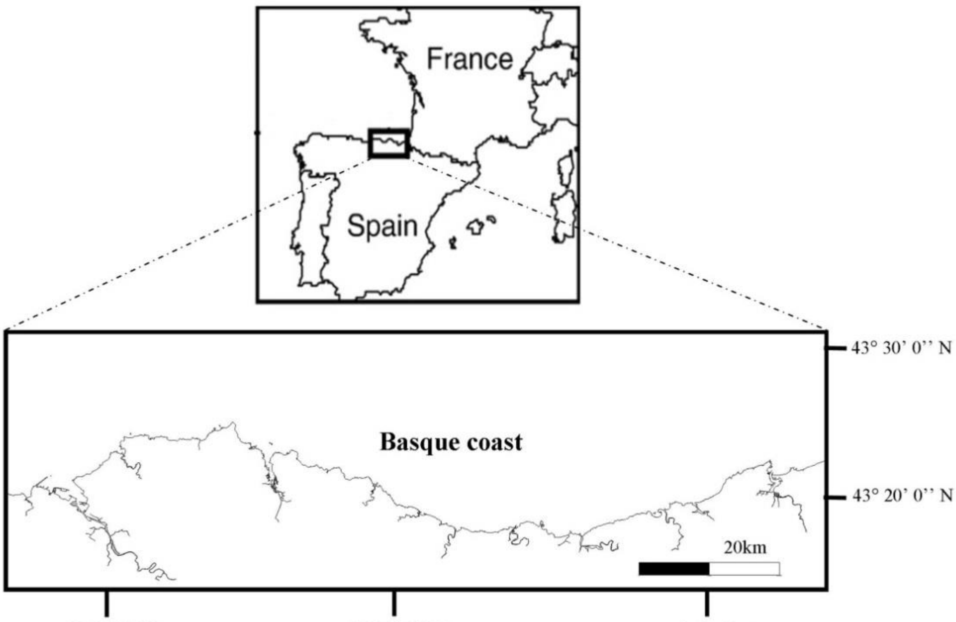

Figure I.14. Map of the study area, the Basque coast (SE Bay of Biscay).

The tide in the Bay of Biscay is semidiurnal, whereby the tidal amplitude ranges from 1 m on neap tides to more than 4.5 m on spring tides. This region is thereby defined as low mesotidal during neap tides and as high mesotidal during spring tides (González et al. 2004). Mineralogically, blenda-galena-pyrite-chalcopyrite paragenesis prevails in the eastern basins, while the western basin is replaced with iron oxides (Belzunce et al., 2004). Therefore, a general increase of metal reference or background levels, especially Fe, Zn, Pb and Cu, are expected in both the dissolved and particulate phases of the aquatic systems (Rodríguez et al., 2006; Tueros et al., 2008).

In comparison with the rest of the North Iberian coast, the Basque region is characterised by its higher population and industrial densities (Prego et al., 2008). Hence, human impact, such as waste and industrial effluents (*e.g.* mining, paper mills and steel factories) are abundant and highly contaminant, especially located in the vicinity of estuaries (Borja et al., 2006b). According to Borja et al. (2006a), nine relevant pressures (*i.e.* nutrients, water and sediment pollution, water abstraction, dredged sediments, shoreline reinforcement, intertidal losses, berths and alien species) affect the Basque coast in both estuaries and coastal waters. However, it is generally acknowledged that recent environmental strategies regarding the treatment and discharge of effluents has led to a significant recovery of the environmental health status of the Basque aquatic systems (Borja et al., 2009). Still, the full extent of this recovery remains to be determined.

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Objectives and hypothesis

The following hypothesis is posed as a basis of this thesis:

"Integrated assessment of environmental pollution using biological effect methods (*i.e.* imposex or histopathology), in combination with tissue and sediment chemistry, might provide holistic and reliable evaluation of the TBT exposure or general health status in a set of sentinel organisms (*i.e.* gastropods, mussels and fishes) within the Basque marine environments"

In order to proof the aforementioned hypothesis, the present work attempts to address the following general objective:

To evaluate if the integrated assessment of environmental pollution using biological effect methods (*i.e.* imposex or histopathology), in combination with tissue and sediment chemistry, could be used to evaluate TBT exposure or general health status in a set of sentinel organisms (*i.e.* gastropods, mussels and fishes) within the Basque marine environments.

The general objective has been subdivided in a series of partial and specific objectives that are shown below and are addressed in the Results and Discussion sections of each chapter:

1. to evaluate the effectiveness of the TBT ban implemented in 2008 through the assessment of imposex levels in two gastropod species (*N. reticulatus* and *N. nitidus*) collected in 2011 along the Basque coast.

2. to develop and validate a species specific semi-quantitative approach based on histopathological indices for the digestive gland and gonad of mussels (*M. galloprovincialis*) collected from five sites with different pollution levels along the Basque coast.

3. to investigate the effects of two critical confounding factors, seasonality and parasites, on histopathological indices of mussels collected along the Basque coast.

4. to evaluate the use of the multi-organ histopathology in gobies, in combination with tissue and sediment chemistry, for environmental risk assessment in the Ibaizabal estuary.

5. to provide a multi-organ (liver, gills, kidney and spleen) histopathological assessment in gobies (*Pomatoschistus* spp.) collected in the Ibaizabal estuary.

6. to test the use of two sentinel fishes (common sole, *S. solea* and European hake, *M. merluccius*) for biomonitoring biological effects within the Basque continental shelf.

7. to adapt, apply and compare two existing histopathological indices in the liver and gonad of common sole and European hake collected along the Basque continental shelf.

8. to evaluate fish histopathology as an indicator of environmental pollution along the Basque continental shelf.

9. to establish how confounding factors may influence the histopathological assessment and subsequent data interpretation in common soles collected along the Basque continental shelf.

10. to determine baseline levels of hepatic and gonad histopathological traits and metal hepatic chemistry in common soles collected monthly over a one-year period along the Basque continental shelf, presumably a pristine area.

Results

The results obtained within this thesis are presented in ***five Chapters***:

Chapter 1: Monitoring the effectiveness of the European tributyltin regulation on the Basque coast by assessing imposex in two gastropod species.

Chapter 2: Development of histopathological indices in the digestive gland and gonad of mussels: Integration with contamination levels and effects of confounding factors

Chapter 3: Multi-organ histopathology in gobies for estuarine environmental risk assessment: A case study in the Ibaizabal estuary.

Chapter 4: Histopathological indices in common sole and European hake for implementation of the European MSFD along the Basque Continental Shelf.

Chapter 5: Histopathological baseline levels and confounding factors in common sole for marine environmental risk assessment.

Chapter 1

Monitoring the effectiveness of the European tributyltin regulation on the Basque coast by assessing imposex in two gastropod species

This Chapter has been published in:

Cuevas, N., García Alonso, J.I., Larreta, J., Rodríguez, J.G., Sariego, C., Zorita, I., 2014. Monitoring the effectiveness of the European tributyltin regulation on the Basque coast (northern Spain) by assessing imposex in two gastropod species (*Nassarius reticulatus* and *Nassarius nitidus*). Chem. Ecol. 30, 636–642.

Summary

Imposex and butyltin body burden were assessed in 2011 along the Basque coast in two gastropod species (*N. reticulatus* and *N. nitidus*) four years after an initial survey in 2007. The aim of this re-survey was to monitor the effectiveness of the European ban on the use of TBT based antifouling paints on ships' hulls (Regulation EC 782/2003). Imposex levels in 2011 were lower than those determined in 2007 at most of the sampling sites. Accordingly, TBT concentrations in the female body burden of *N. reticulatus* varied from 43 to 250 ng Sn g^{-1} in dw in 2011, which was a lower maximum than in 2007. Nevertheless, the results for the butyltin degradation index suggest that there have been recent inputs of TBT within the two main Basque harbours. Overall, the legislative measure is contributing to the reduction of TBT effects on the Basque coast, although its presence is still evident.

1. Introduction

The chemical biocide TBT is leached to the marine environment from antifouling paints used on vessels and other immersed structures. TBT-based antifouling paints widely used in vessels since the 1970s, have been considered the most cost-effective option (Terlizzi et al., 2001). However, TBT is classified among the most toxic xenobiotics introduced by humans into marine waters (Goldberg, 1986). Hence, its toxicity is reflected in a wide range of organisms, causing sublethal and lethal damage in microorganisms, invertebrates and vertebrates (Alzieu, 1996). One of the most studied effects is imposex, *i.e.* imposition of male sexual characters, such as a penis and vas deferens, onto female dioecious gastropods, which is used as a biomarker of TBT exposure (ICES, 2004) and to a lesser degree of triphenyltin (TPT) exposure (Barroso et al., 2002).

In order to control the use of this biocide, at least partly, industrialised countries started to restrict the use of TBT in the 1980s (*e.g.* Directive 89/677/EEC). But the inefficiency of this partial ban was proved during the 1990s (Ruiz et al., 2008). Consequently, in 2001 the International Maritime Organization (IMO) considered an international agreement on vessel antifouling systems damage control (Anti-Fouling System agreement, AFS) and in the EU this regulation became stricter in 2003 with the Regulation (EC) 782/2003, which declared a total ban on the application of TBT-based antifouling paints in new ship coatings after 1st July 2003. Likewise, since 1st January 2008, carrying a TBT-based paint in vessels sailing to or from European ports is completely restricted. These legislative measures have promoted several studies to assess whether the above mentioned bans are contributing to the recovery of the quality of the marine environment (*e.g.* Barroso et al., 2002; Pavoni et al., 2007). Among these studies, a survey on imposex was carried out in 2007 on the Basque coast (northern Spain), an area with a high density of ports and marinas (Rodríguez et al., 2009a; 2009b). This research established a baseline prior to the 2008 European ban of TBT antifouling paints. Hence, the aim of the present work is to assess the imposex levels in *N. reticulatus* and *N. nitidus* along the Basque coast in 2011 in order to evaluate the effectiveness of the above mentioned bans.

2. Material and methods

2.1. Sampling and imposex assessment

The gastropods *N. reticulatus* (21 sites) and *N. nitidus* (10 sites) were collected with baited hoop nets during March-July 2011 (Figure 1.1). The animals were transported to the laboratory and maintained in aquaria with seawater (33 psu), for 24h. Only adult animals, those presenting a white columellar callus and teeth on their outer lip, were evaluated. Before shell removal, gastropods were narcotised in two steps: (*1*) they were submerged in a mixture of 1:1 (v/v) of seawater (33 psu) and $MgCl_2$ (7% w/v) aerated solution for 1 h and then (*2*) individuals were transferred to a $MgCl_2$ (7% w/v) solution for 30 minutes (Fernández et al., 2007). Afterwards, the estimation of following imposex parameters were determined: imposex frequency (I%); Vas Deferens Sequence Index (VDSI) *sensu* Stroben et al. (1992); Relative Penis Length Index (RPLI) (calculated as (mean female penis length×100)/mean male penis length); and percentage of sterile females (S%). These imposex parameters and the selection of sampling sites were carried out as in the first survey in 2007 performed by Rodríguez et al. (2009a; 2009b) (see also Cuevas et al., 2011). In order to avoid differences in imposex expression both surveys were conducted at the same period of the year (Barroso and Moreira, 1998).

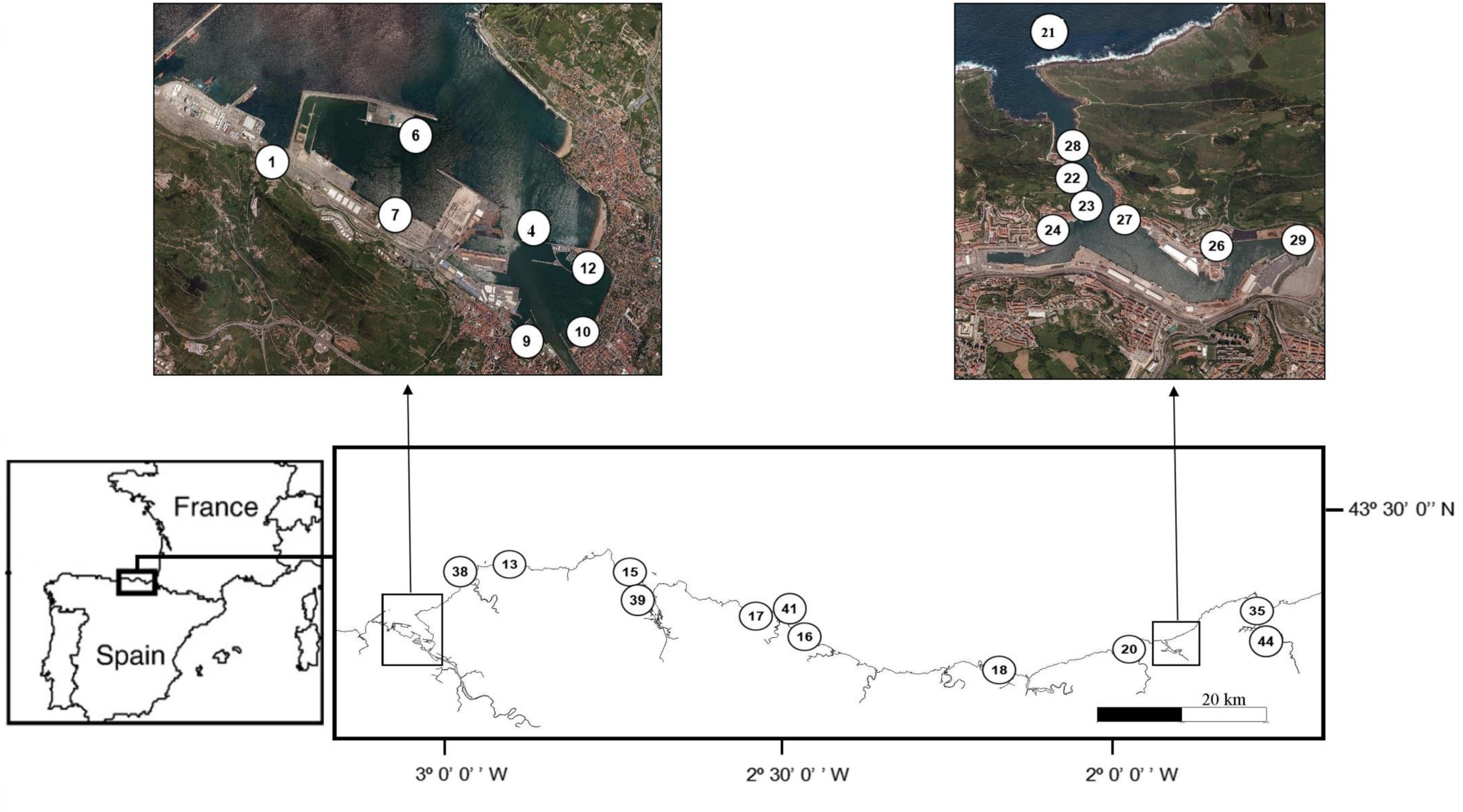

Figure 1.1. Location of the sampling sites in the Basque coast, northern Spain.

2.2. Organotin analyses

The contents of monobutyltin (MBT), dibutyltin (DBT) and TBT were measured in whole tissues of pooled females of *N. reticulatus* collected from eight sampling sites located within the two main Basque ports (Figure 1.1). Tissue samples were lyophilised for 49 h (Lyobeta-15, Telstar). Lyophilised tissue samples were spiked with ^{119}Sn-enriched spike solution and then 5 ml of a mixture of acetic acid and methanol (3:1, w:w) were added to a weighted sample in a glass vial. These vials were introduced into a thermostatic bath at 37 ºC for at least 2 h. Subsequently, 300 ml of the extractant was ethylated by adding 3 ml of acetate buffer to adjust the pH to 5.4 and finally, 200 ml of a 2% w/v sodium tetraethylborate in 0.2 M NaOH were added. Further, 1ml of hexane was added and subsequently 1ml of the organic extract was injected in the GC-ICP-MS. (Model 6890 coupled to a HP-7500ce ICP-MS). The validation of this methodology was evaluated with mussel certified reference material CRM 477 (Community Bureau of Reference (BCR), Commission of the European Communities) (further information García Alonso et al., 2002).

The Butyltin Degradation Index (BDI) was calculated as ([MBT] + [DBT]/[TBT])) to determine the predominance of TBT over its metabolites.

2.3. Statistical analyses

Normality of data was evaluated by Shapiro-Wilk test. Differences between 2007 and 2011 in imposex levels (I%, RPLI, VDSI and S%) were tested using chi-square test and Mann-Whitney U test. Regressions between TBT and imposex levels (RPLI and VDSI) were also assessed. All the statistical analyses were performed at $p < 0.05$ or $p < 0.01$ using the Statgraphics Plus 5.5 software.

3. Results and discussion

3.1. Imposex assessment

Out of the 616 *N. reticulatus* assessed, 74.2% were females and 25.8% were males. 393 *N. nitidus* specimens were studied, of which 51.1% were females and 48.9% males. Mean shell length ranged from 10.8 to 32.6 mm for *N. reticulatus* and from 16.8 to 30.3 mm for *N. nitidus*. Imposex parameters are summarised in Table 1.1. Imposex-affected females were found at all sampling sites in 2011, except at site 21. Imposex levels and indices for *N. reticulatus* ranged between 85 and 100% for I% affected females, 0.8 and 79.4% for RPLI, 1.6 and 4.0 for VDSI, and 0 and 25% for the incidence of sterile females. For *N. nitidus* the values ranged between 80 and 100% for I% affected females, 14.1 and 72.1% for RPLI, 1.5 and 3.9 for VDSI, and 0 and 26.7% for the incidence of sterile female. In both species, imposex affected females mainly presented a-type VDS stages showing simultaneous penis development. The spatial distribution of imposex levels in 2011 confirmed that harbours are hot spots of TBT pollution, in particular Pasaia (sampling sites 22 – 29).Comparing the imposex levels of the two main Basque harbours (*i.e.* Pasaia and Bilbao), higher imposex levels were recorded in Pasaia (RPLI, 32.44 – 78.13%; VDSI, 2.36 – 4.00) than in Bilbao (RPLI, 0.82 – 50.50%; VDSI, 1.17 – 3.30). This pattern is also reflected in the results of the 2007 survey carried out by Rodríguez et al. (2009a; 2009b). Although Bilbao presented higher traffic of large vessels than Pasaia, this latter is a confined harbour with low hydrological dynamic owing to its narrow estuarine mouth (Rodríguez et al., 2009a). In middle-sized to small port areas, imposex levels were generally lower, except in three small ports: Zierbena (site 1), Getaria (site 18) and Donostia (site 20), where higher levels of imposex were recorded in 2011. The absence of imposex-affected females in Murgita (site 21) is in

Table 1.1. Comparison of I%, RPLI, $VDSI_4$ *sensu* Stroben et al. (1992) and S% along the Basque coast before (2007) and after (2011) the European TBT regulation. Asterisks indicate statistically significant differences between campaigns (Chi-square test and Mann-Whitney U test, *: $p < 0.05$; **: $p < 0.01$). M: marina; F: fishing fleet; S: shipyard; H: harbour; O: open berth area.

N. reticulatus			I%		RPLI		$VDSI_4$		S%	
Location	Sampling site	TBT source	2007	2011	2007	2011	2007	2011	2007	2011
Zierbena	1	M,F	72	96*	17.1	54.5*	1.6	3.2*	0	7.7
Bilbao	4	H,O	100	95	23.0	8.8*	3.1	1.3*	0	5.0
Bilbao	6	H,O	100	96	4.4	2.2*	1.8	1.3*	0	0
Bilbao	7	H.O	100	100	15.7	0.8*	2.6	1.2*	2.2	0
Bilbao	9	H,F,S	100	100	53.6	50.5	3.6	3.3	0	4.3
Bilbao	12	M	100	100	45.0	5.5*	3.9	1.6*	0	0
Armintza	13	M,F	73	100**	54.6	22.2*	2.9	2.0	9.1	0
Bermeo	15	H,F,S	100	100	76.7	17.7*	3.7	2.2*	14.3	9.5
Mutriku	16	M	100	100	72.1	33.7*	3.7	3.1*	0	3.3
Lekeitio	17	M,F	78	94	44.9	20.4	2.7	1.9	0	0
Getaria	18	M,F	75	97*	61.5	79.4	3.0	3.6	0	25.0
Donostia	20	M,S	83	100*	38.3	59.3	2.0	3.7*	0	0
Murgita	21	N	0	0	0	0	0	0	0	0
Pasaia	22	M,O	100	100	68.8	64.7	4.0	3.5*	8.7	5.3
Pasaia	23	M,O	100	100	70.1	50.0*	3.9	3.5*	10.5	11.1
Pasaia	24	H,F	100	100	67.7	33.3*	3.9	3.4*	9.5	7.7
Pasaia	26	H,S	100	100	92.0	78.1*	4.0	4.0	14.3	9.5
Pasaia	27	H,O	100	100	85.9	60.9*	4.0	3.6*	0.0	8.7
Pasaia	28	O,S	97	100	46.9	32.4*	3.6	2.4*	6.3	0
Pasaia	29	H	100	100	75.0	70.8	4.0	3.8	4.5	16.7
Hondarribia	35	M	100	85**	25.1	4.3*	2.7	1.6*	0	0
N. nitidus			I%		RPLI		$VDSI_4$		S%	
Location	Sampling site	TBT source	2007	2011	2007	2011	2007	2011	2007	2011
Bilbao	9	H,F,S	100	100	71.6	32.8*	4.0	2.4*	0	4.2
Bilbao	10	M	100	100	45.4	36.8	3.4	2.8	0	4.0
Bilbao	12	M	100	100	57.9	23.2*	4.0	2.4*	0	0
Getaria	18	M	100	100	91.0	72.1*	4.0	3.3*	17.0	6.7
Pasaia	26	H,S	100	100	86.1	72.1	4.0	3.9	13.0	10.0
Pasaia	29	H	100	100	68.0	48.9*	4.0	3.5*	23	26.7
Hondarribia	35	M	100	85	55.3	15.2*	4.0	1.6*	7.0	0
Plentzia	38	M	100	100	24.7	14.1	2.2	1.7	0	0
Mundaka	39	M	100	100	82.4	42.9*	4.0	3.0*	13.0	23.1
Ondarroa	41	H,F	100	100	89.4	52.0*	4.0	2.6*	17.0	18.8
Hondarribia	44	O	89	80	16.5	19.7	1.6	1.5	0	0

accordance with the absence of TBT sources in surrounding waters and the predominant hydrodynamic characteristics of this site, which is located in a coastal area (*i.e.* offshore). Sterility data showed very low prevalences in almost all sampling sites, thus this variable was unable to discriminate temporal and spatial differences.

In order to determine the effectiveness of the TBT ban on the Basque coast, imposex levels in *N. reticulatus* and *N. nitidus* females obtained in 2011 were compared with those recorded in 2007 by Rodríguez et al. (2009a; 2009b). These data are summarised in Table 1.1. A statistically significant decrease in RPLI in *N. reticulatus* was found at 14 sampling sites. This decreasing pattern was observed in small, mid and large harbours. At Zierbena (site 1), despite being a port close to open sea and with absence of large vessels, a statistical significant increase was found, while no statistically significant differences were recorded at the other seven sampling sites (Table 1.1). A statistically significant decrease in RPLI in *N. nitidus* was detected at seven sites and no statistically significant differences were determined at the other four sampling sites. Similar results were found for VDSI (Table 1.1). The increase in imposex levels recorded in *N. reticulatus* from Zierbena (site 1) could be related to the dredging activities carried out in this area between the two sampling periods, which involved the re-suspension of polluted sediment and the possible increase in TBT bioavailability. The increase in VDSI found in *N. reticulatus* at Donostia (site 20) is possibly associated with the paint flakes that are generated in the frequent cleaning of yachts and boats in this area. As these flakes may originate from antifouling paints with TBT this hypothesis requires further research. At Getaria (site 18) a significant increase was determined in the percentage of imposex-affected *N. reticulatus* females, but no significant increase was found for RPLI and VDSI. Moreover, significant decreases in RPLI and VDSI were recorded in *N. nitidus* at this sampling site (Table 1.1). Both species, *N. reticulatus* and *N. nitidus*, have a relative similar response to TBT levels along the Basque coast (Rodríguez et al., 2009b). In Bilbao (sites 9 and 12), Pasaia (site 26 and 29) and Hondarribia (site 35) both species showed a decreasing trend (Table 1.1). Therefore, the complementary use of both species in monitoring studies along the Basque coast could provide wider spatial coverage.

3.2. Organotin analyses

Organotin compounds were determined in eight samples of *N. reticulatus* females collected in 2011 at the two main Basque ports. These compounds were also analysed in the first survey (2007). Hence, in 2011 TBT body burden concentrations ranged between 43 and 250 ng Sn g^{-1} in dw, whereas in 2007 they ranged between 14 and 405 ng Sn g^{-1} in dw (Figure 1.2) (Rodríguez et al., 2009a). The highest TBT levels in female body burden were recorded in Pasaia harbour in accordance with the highest imposex values. However, the maximum TBT value decreased considerably (by nearly half) between 2007 and 2011, although TBT concentrations increased at some sampling sites during the last survey. The relative concentrations of TBT, DBT and MBT showed similar patterns at all the sampling sites (Figure1.2). In both surveys all the sampling sites had BDI values above 1 except Zierbena (site 1) and Bilbao (site 4) in 2011 (Figure 1.2). Generally, BDI values below 1 correspond to recent inputs of TBT (Díaz et al., 2008), *i.e.* lower BDI values can indicate fresher inputs of TBT (Barroso et al., 2002). The values recorded in 2007 were higher than those from 2011 (Figure 1.2). This decrease in BDI values may indicate that there were new inputs of TBT between the two surveys. Similar results were found previously in *Nucella lapillus* in NW Spain between 2000 and 2005, but not in *N. reticulatus* (Barroso et al., 2002; Ruiz et al., 2010).

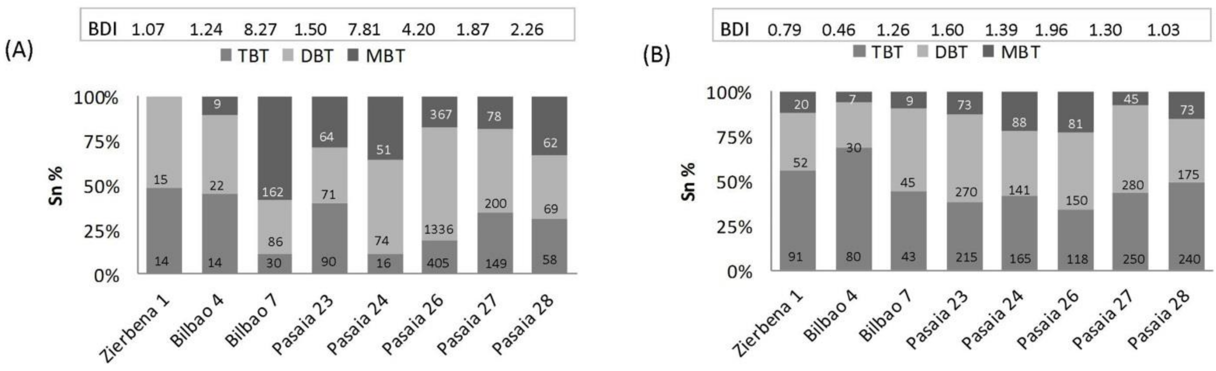

Figure 1.2. Absolute (indicated with numbers within the bars in ng g^{-1}), relative concentration (%) of TBT, DBT and MBT, and BDI in whole female body burden in (*A*) 2007 and (*B*) 2011 at each sampling site. Sn concentrations are given on a dw basis.

3.3. Data integration

Positive and logarithmic regressions between TBT whole female body burden and imposex levels (RPLI and VDSI) were found in both years in *N. reticulatus*. This logarithmic relation between both variables is common in this species (Rodríguez et al., 2009a), but significant regressions ($p < 0.05$) were only recorded between RPLI and TBT in 2007 and between VDSI and TBT in 2011 (Figure 1.3). Nevertheless, in 2011 lower RPLI and VDSI values were found for a similar TBT body burden (Figure 1.3).

Thus, the lower BDI values registered in 2011 with respect to 2007 could represent a recent input of TBT, probably related to dredging activities and/or desorption from sediments (Barroso et al., 2002). However, this input is not reflected at the biological level because lower imposex levels were generally observed in 2011 compared to 2007. One explanation could be related to the fact that the development of male characters in females requires a moderate time period of around several months, at least in *N. reticulatus* (Rodríguez et al., 2010). Accordingly, it has been stated that the required time to express biological effects after the exposure to toxicants depends on several factors (*e.g.* Gaion et al, 2013; Mesarič et al., 2013).

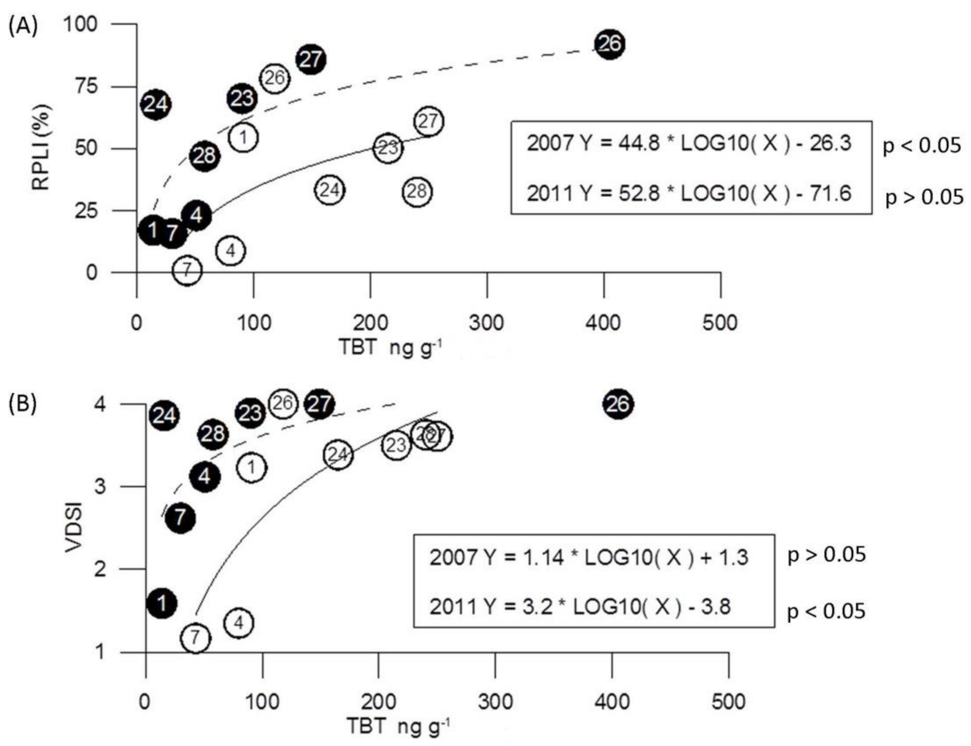

Figure 1.3. (*A*) Relationship between RPLI and TBT whole female body burden. (*B*) Relationship between $VDSI_4$ *sensu* Stroben et al. (1992) and TBT whole female body burden. Note: black circles represent data of 2007, whilst white ones reflect data of 2011. Labels relate to sampling site number (see Table 1.1). Concentration of TBT is given as Sn on a dry weight basis. Note: statistical significance should be interpreted carefully since parametric requirements are not fulfilled.

Following the Assessment Class Criteria for imposex given in the OSPAR Coordinated Environmental Monitoring Programme (CEMP) (OSPAR, 2004), the percentage of sampling sites for *N. reticulatus* within each class from 2007 to 2011 were: A – B classes (VDSI < 0.3) 4.8% in both years, C class (0.3 < VDSI < 2.0) from 9.5 to 28.6%, D class (2.0 < VDSI < 3.5) from 28.6 to 42.9%, and within E class (VDSI > 3.5) from 57.1 to 23.8%. In the case of *N. nitidus* the sampling site classification was: A – B classes 0% in both years, C class from 10 to 30%, D class from 20 to 60% and finally E class from 70 to 10%. Therefore, according to the OSPAR classification, there was an improvement in the ecological impact of TBT although almost all sampling sites had higher values than the Ecological Quality Objective (*i.e.* VDSI < 0.3) (OSPAR, 2007).

4. Conclusions

In conclusion, although the imposex levels improved after the implementation of the European TBT ban within the studied area, the BDI results indicate the possibility of fresh TBT inputs. These recent TBT inputs could have come from sediment desorption, dredging activities or illegal use of prohibited antifouling paints. However, further research is required to determine the effectiveness of the European TBT regulation (EC 782/2003).

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Chapter 2

Development of histopathological indices in the digestive gland and gonad of mussels: Integration with contamination levels and effects of confounding factors

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Summary

Bivalve histopathology has become an important tool in aquatic toxicology, having been implemented in many biomonitoring programmes worldwide. However, there are various gaps in the knowledge of many sentinel organisms and the interference of confounding factors. This work aimed (*1*) to develop a detailed semi-quantitative histopathological index of the digestive gland and gonad of the *M. galloprovincialis* mussel collected from five sites contaminated with distinct patterns of organic and inorganic toxicants along the Basque coast and (*2*) to investigate whether seasonal variability and parasitosis act as confounding factors. A total of twenty-three histopathological alterations were analysed in the digestive gland and gonad following a weighted condition index approach. The alterations were integrated into a single value for a better understanding of the mussels' health status. The digestive gland was consistently more damaged than the gonad. Mussels from the most impacted sites presented the highest Cd and Cu bioaccumulation levels and endured the most significant deleterious effects, showing inflammation-related alterations together with digestive tubule atrophy and necrosis. Neoplastic diseases were scarce, with only a few cases of fibromas (benign neoplasia) and one case of haemocytic neoplasia (malignant neoplasia). In contrast, in moderately or little impacted sites, contamination levels did not cause significant tissue damage. However, parasites contributed to overestimating the values of histopathological indices (*i.e.* more severe tissue damage) in mussels from little impacted sites, whilst the opposite occurred in mussels from highly polluted sites. Accordingly, inter-site differences were more pronounced in autumn when natural physiological responses of advanced maturation stages did not interfere in the histological response. In conclusion, although seasonal variability and parasitosis mask the response of histopathological indices, this biomonitoring approach may provide

good sensitivity for assessing the health status of mussels if fluctuations of these confounding factors are considered.

1. Introduction

In the EU, the MSFD (Directive 2008/56/EC), introduced a series of criteria ("Descriptors") that must be addressed in order to achieve GES of marine habitats. As such, the MSFD stipulates that the monitoring of marine ecosystems is to be endeavoured regionally, in face of the broad biogeographical diversity of the European coastline, albeit the need for some degree of standardisation of biomonitoring procedures (see the reviews by Borja et al., 2010; Lyons et al., 2010).

The effects of anthropogenic contaminants on the biota are included in Descriptor 8 of the MSFD. However, the choice for adequate biological endpoints and sentinel organisms is challenging on its own and relies on solid knowledge of the aetiology of stressor-induced adverse effects and the specific life-history traits that modulate them (Ruiz et al., 2011; van der Oost et al., 2003). For instance, the expert scientific groups of ICES, such as the WGBEC and the SGIMC, assist in the development of assessment tools under Descriptor 8 premises within the North East Atlantic maritime region (Davies and Vethaak, 2012). Since the establishment of the Mussel Watch Program in the mid-1970s (Goldberg, 1975), mussels have been extensively used as a sentinel organism of pollution in marine coastal biomonitoring programmes (Bignell et al., 2011; Brenner et al., 2012; Costa et al., 2013; Zorita et al., 2007). Mussels (Bivalvia: *Mytiloidea*) hold many advantageous characteristics since they have a wide geographic distribution (with potential implications for standardised analyses); they are sedentary filter feeders (thus prone to be affected by their immediate surroundings); have a relatively simple anatomy and physiology; and are acknowledged as being sensitive to a range of pollutants (Bignell et al., 2011; Brenner et al., 2012; Cajaraville et al., 2000; Widdows et al., 2002).

Defining histopathological lesions and alterations in wild organisms has been considered as one of the most important approaches to assess pollution-driven adverse effects to organisms in biomonitoring studies. Histopathological biomarkers are considered to be high-value endpoints since they may provide direct information on the organisms' health status (Adams and Sonne, 2013; Bignell et al., 2008; ICES, 2004). Advances in semi-quantitative approaches (or even fully quantitative, in a few cases) have been equipping histopathology with an important statistical power that is critical in biomonitoring (Bignell et al., 2011; Costa et al., 2013; Marigómez et al., 2013). Histopathological indices that integrate different histopathological alterations of a target organ into a single value have already been developed in different fish species (Bernet et al., 1999; Costa et al., 2009, 2011; van Dyk et al., 2007) and, to a lesser extent, in invertebrates, such as clams (Costa et al., 2013). However, to our knowledge, there is no detailed histopathological index that integrates and unifies all the lesions recorded in mussels, one of the proposed sentinel species for the biomonitoring of EU coastal waters within the scope of the MSFD.

Nevertheless, histopathological studies may be impaired by several factors that call for in-depth knowledge of the sentinel organisms' specific biological features, from season-related changes (including gonad development) and parasites to the identification of specific microanatomical traits (Bignell et al., 2008; Garmendia et al., 2010; Kim et al., 2008). In fact, the latter is regarded as a major drawback in histopathological appraisals of invertebrates since these are a long way off benefitting from the level of histological knowledge as their vertebrate counterparts (see Au, 2004, for a review).

This work is aimed at (*1*) developing and validating a species-specific semi-quantitative approach based on histopathological indices for the digestive gland and gonad of mussels (*M. galloprovincialis*) collected from five sites contaminated with distinct patterns of organic and inorganic toxicants along the Basque coast; and (*2*) investigating the effects of two critical confounding factors, seasonality and parasites, on mussel health status and how these may hamper biomonitoring procedures.

2. Material and methods

2.1. Sampling area and sample collection

The Basque coast, in particular its estuaries, has been receiving special attention in marine environmental assessment due to its historical concentration of heavy industry and dense human settlement (Borja et al., 2006). For many years, the ecological status of the Basque estuaries deteriorated, especially because of the direct dumping of untreated domestic and industrial wastewaters (Cearreta et al., 2004). However, it is generally acknowledged that recent environmental strategies regarding the treatment and discharge of effluents has led to a significant recovery of the environmental status along the Basque coast (Borja et al., 2009). Yet, the full extent of this recovery remains to be determined. Hence, this study focused on five different sites along the Basque coast: Getxo, Gorliz, Mundaka, Pasaia and Hondarribia (Figure 2.1). These estuarine environments are classified into two major groups, according to their geomorphological and hydrological characteristics (Borja et al., 2004): Gorliz and Mundaka are estuaries dominated by marine influence with a high percentage (> 75%) of intertidal area; while Getxo, Pasaia and Hondarribia are subtidal estuaries. Getxo and Pasaia boast important and industrialised harbours on the North Iberian coast, presenting a deteriorated ecological status characterised by low oxygen rates, disappearance of fauna and significant levels of sediment pollution (Borja et al., 2006). Conversely, Mundaka is located in the Urdaibai Biosphere Reserve, which is considered one of the best preserved estuaries of the Basque coast (Marigómez et al., 2013; Orbea et al., 2002). This estuary is mainly important for leisure and tourism, although some industrial activities can be found further upstream, such as metallurgic and shipyard facilities. Gorliz lacks significant contamination hotspots, being an area exposed to moderate waves (Orbea et al., 2002; Solaun et al., 2013). Lastly, Hondarribia is located off the mouth of Bidasoa estuary, a highly populated area that has been recovering its ecological status due to the recent implementation of a sanitation plan that included a treatment plant and respective submarine outfall (Solaun et al., 2013).

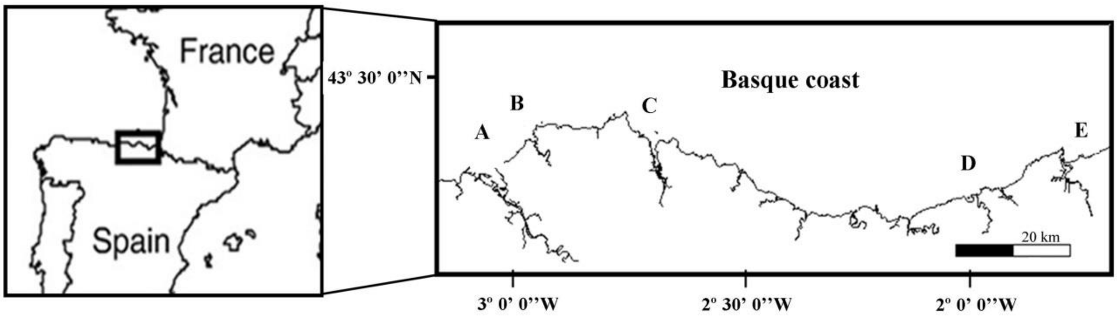

Figure 2.1. Map of the study area showing the sampling sites: (*A*) Getxo, (*B*) Gorliz, (*C*) Mundaka, (*D*) Pasaia and (*E*) Hondarribia.

In each locality, two sediment samples were collected by hand in intertidal areas and with Van Veen or Day grabs in subtidal areas every winter between 2009 and 2012. Then, one sediment sample was stored at 4 ºC for sediment characterisation, whereas the other sample was frozen at -20 ºC for contaminant determination. Additionally, adult mussels (3.5 – 5.5 cm in length) were sampled at each sampling site (n = 40) during low tide in the four seasons of a full year, from spring 2012 to winter 2013. The specimens were transported in sea water to the laboratory until subsequent histological and chemical analyses.

2.2. Sediment characterisation and contamination levels

Granulometric analyses were conducted on dried sediments (60 ºC, 24 h) by running samples through a column of sieves (gravel, > 2 mm; sand, 2 – 63 mm and mud, < 63 mm) according to Holme and McIntyre (1971). Mean grain size values were determined with GRADISTAT software (Blott and Pye, 2001). Techniques used in the determination of the grain size distribution of the samples varied depending on the fine content of the sediments. Samples with low percentage of fine sediment (< 10%) were determined by dry sieving according to Folk (1974); while samples with high content of fine sediments (> 10%) were determined by Laser Diffraction Particle Size Analyser (LDPSA); due to the underestimation of the finest fraction, mud content was transformed following the method proposed by Rodríguez and Uriarte (2009).

Metals (Cd, Cr, Cu, Hg, Ni, Pb and Zn) were measured in triplicate following acid digestion of dry fine sediment samples (< 63 mm) in a microwave (CEM MARS 5 Xpress) by Atomic Absorption Spectrometry, AAS (PerkinElmer AAS AAnalyst 800). Cd was analysed by Transversely Heated Graphite Atomizer (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 vapor method. Analytical accuracy was tested using a certified reference material (PACS-2, National Research Council of Canada (NRC)) and the measured values were found to be within the certified range. Recoveries for the certified metals were: 92% Cd; 70% Cr; 95% Cu; 95% Hg; 99% Ni; 100% Pb and 95% Zn.

For organic toxicants, PCBs (28, 52, 101, 105, 118, 138, 153, 156 and 180) and PAHs (fluorene, naphthalene, anthracene, dibenz(a,h)anthracene, acenaphthene, acenaphthylene, phenanthrene, pyrene, chrysene, benzo(e)pyrene, benzo(g,h,i)perylene, fluoranthene, benzo(a)anthracene, benzo(b)fluoranthene, benzo(a)pyrene, indeno (1,2,3-cd)pyrene), sediment sample (5 – 10 g) extracts were pre-concentrated with a mixture of solvents (pentane:dicloromethane; 50:50) by accelerated solvent extraction, ASE (Dionex ASE200 system). 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). Mean recoveries for almost all the certified PAHs were between 80 and 90%. Indeno[1,2,3-cd]pyrene and benzo[ghi]perylene showed lower recovery rates of between 60 and 70%. Mean recoveries, for almost all the certified PCBs, were between 85 – 110%.

To assess the toxicological significance of sediments, contaminant levels were compared to the TEL and PEL quality guidelines for sediments proposed by MacDonald et al. (1996). The risk of causing adverse biological effects was determined for each sediment according to the Sediment Quality Guideline Quotients (SQG-Qs) described by Long and MacDonald (1998). Additionally, sediments were scored according to the risk of causing adverse biological effects as proposed by MacDonald et al. (2004): not impacted (< 0.1); moderately impacted (0.1 – 1) and strongly impacted (> 1).

Medians of granulometry and SQG-Qs of sediments collected between 2009 and 2012 were calculated in order to characterise the sediment matrix of each estuarine area.

2.3. Metal bioaccumulation levels

Metals (Cd, Cu, Hg, Pb and Zn) were measured in a pool of 20 mussels per season for each sampling site. Mussels were homogenised and digested in 10 ml of concentrated HNO_3 in a microwave oven (MARS 5 Xpress CEM Corporation Instrument). Metal content was then analysed by AAS (using a AAS800 model apparatus, Perkin Elmer). Total mercury was determined using the cold vapour technique and the remaining metals (Cd, Cu, Pb and Zn) by graphite furnace equipped with a Zeeman background correction device. The validation of the analytical procedure was carried out using mussel tissue as certified reference material (278R, BCR). The mean recoveries were in the range of 80 – 90% for all of the metals, throughout the study.

2.4. Histology and histopathological procedure

Fresh portions of digestive gland and gonads of 20 mussels were dissected per season for each sampling site and were immediately fixed in Davidson's solution (48 h at 4 ºC). Samples were dehydrated through a progressive series of ethanol, intermediately infiltrated by xylene and embedded in paraffin. Tissue sections (3 − 5 µm) were obtained using a rotary microtome (Microm HM 350 S), stained with haematoxylin and eosin (H&E), clarified in xylene and mounted with a resinous medium. Lesions and alterations were investigated using an Olympus BX60 microscope.

Firstly, a qualitative description of both organs was performed and then the prevalence of histopathological alterations was calculated as (number of cases/total cases analysed) × 100 per sampling site and season. Afterwards, a semi-quantitative approach was estimated for each organ, per individual, integrating the main recorded alterations into a single index. The histopathological indices for the digestive gland and the gonad were based on the weighted indices originally proposed by Bernet et al. (1999) for fish and then adapted by Costa et al. (2013) for clams. Briefly, the histopathological assessment considered the concepts of biological significance (weight) of the alteration to which a value of between 1 (minimal significance) and 3 (highest severity) and dissemination degree (score), ranging from 0 (feature or alteration not observed) to 6 (diffuse) were attributed. The histopathological indices were calculated through the formula [1], as described by Costa et al. (2013):

$$Ih = \frac{\sum_{1}^{j} wj\ ajh}{\sum_{1}^{j} Mj} \qquad [1]$$

Where *Ih* is the histopathological index for the individual *h*; *wj* the weight of the *jth* histopathological alteration; *ajh* the score value selected for the jh alteration and *Mj* is the attributable maximum (weight × maximum score) for the *jth* alteration. The denominator of the equation standardises the indices to a value between 0 and 1, permitting comparisons between different conditions such as different organs, sampling sites or campaigns.

For each organ, the histopathological alterations were divided into three reaction patterns and afterwards a global histopathological index was estimated with and without parasitosis. In Table 2.1, the lesions comprised within each reaction pattern for both organs are summarised. Due to the lack of literature on similar research in mussels, the weights of alterations were based on previous work on clams (Costa et al., 2013) and fish (Bernet et al., 1999; Costa et al., 2011). Specific weights for infections by different parasites were attributed following their known biological significance (see da Silva et al., 2011). The accuracy of the histopathological analyses was checked by a series of blind reviews.

Apart from the histopathological alterations, six developmental stages were distinguished in mussel gonads according to Seed (1969), and gonad index (GI) values ranging from 0 to 5 were assigned to each developmental stage as in Cajaraville et al. (1992). The median GI was then estimated for males and females of each sampling group.

Table 2.1. Observed pathologies in the digestive gland and the gonad and their importance weight (w).

	Reaction pattern	**Alteration**	**w**
Digestive gland	Tubule alterations	Digestive tubule atrophy	2
		Necrosis	3
		Brown cells	1
		Lipofuscin aggregates	1
	Intertubular alterations	Haemocytic infiltration	2
		Necrosis	3
		Fibrosis	2
		Granulocytomas	2
		Brown cells	1
		Lipofuscin aggregates	1
	Parasitosis	*Nematopsis*	3
		Mytilicola intestinalis	2
		Other parasites	2
Gonad	Interlobular alterations	Haemocytic infiltration	1
		Fibrosis	2
		Granulocytomas	2
		Lipofuscin aggregates	1
		Brown cells	1
	Intralobular alterations	Necrosis	3
		Haemocytic infiltration	1
		Lipofuscin aggregates	1
		Oocyte atresia/hermaphroditism	3
	Parasitosis	*Nematopsis*	3

2.5. Statistical analyses

Statistics were computed with Statgraphics (StatPoint Technologies). The normality and homoscedasticity of data were analysed through the Kolmogorov-Smirnov and Levene's tests, respectively. Following invalidation of at least one of the assumptions to perform parametric analyses of variance, the non-parametric tests (the Kruskal-Wallis followed by the Mann-Whitney U and Wilcoxon's Matched-Pairs tests) were applied for pairwise comparisons. Correlations between metal bioaccumulation and histopathological indices were assessed through the non-parametric Spearman's rank-order correlation R statistic. Finally, after the verification of non-linearity between means and variances, the discriminant analyses were conducted to assess the most significant variables to account for inter-site variation. All analyses were carried out at $p < 0.05$ significance level.

3. Results

3.1. Sediment characterisation and contamination levels

Granulometry and toxicological significance measured through SQG-Qs of sediments collected between 2009 and 2012 in the Basque estuaries is summarised in Table 2.2. Granulometry data showed that Mundaka and Hondarribia were sandy estuaries; while Getxo, Gorliz and Pasaia were mainly muddy. Pasaia presented sediments strongly impacted by metals (SQG-Q > 1), where Cu, Pb and Zn revealed the highest toxicological significance.

However, Getxo showed sediments strongly impacted by organic toxicants, where low molecular weight PAHs were the most toxic. The remaining sites, Gorliz, Mundaka and Hondarribia, showed sediments moderately impacted by metals and non-impacted by organic compounds, except those of Gorliz which yielded moderate risk for organic compounds.

Table 2.2. Median of granulometry, and metal and organic SQG-Qs of sediments collected every winter from 2009 to 2012 in the five sampling sites along the Basque coast. N: non-impacted; M: Moderately impacted; S: strongly impacted.

	Parameters	Getxo	Gorliz	Mundaka	Pasaia	Hondarribia
Granulometry (%)	Gravel	0.1	0.5	0.3	0.00	0.1
	Sand	16.7	22.7	99.9	17.1	99.4
	Mud	83.2	76.8	0.07	82.9	0.6
SQG-Qs metals	Cd	0.26	0.04	0.02	0.32	0.05
	Cr	0.36	0.14	0.08	0.34	0.25
	Cu	0.64	0.23	0.05	1.34	0.16
	Hg	1.11	0.12	0.08	0.71	0.07
	Ni	0.88	0.65	0.58	0.97	0.84
	Pb	0.97	0.48	0.16	1.39	0.17
	Zn	0.94	0.41	0.20	2.09	0.32
	Total metals	0.74 (M)	0.29 (M)	0.17 (M)	1.02 (S)	0.26 (M)
SQG-Qs organic compounds	PCBs	1.41	0.12	0.10	1.70	0.10
	Low PAHs	1.63	0.09	0.04	0.32	0.01
	High PAHs	1.16	0.40	0.07	0.21	0.00
	PAHs	1.01	0.23	0.04	0.20	0.00
	Total organic compounds	1.30 (S)	0.21 (M)	0.06 (N)	0.61 (M)	0.03 (N)

3.2. Metal bioaccumulation levels

Metal bioaccumulation levels determined in four seasons in mussels from the five sampling sites of the Basque coast are summarised in Table 2.3. Although spatial variations were not very pronounced, mussels from Getxo and Pasaia presented slightly higher bioaccumulation levels of Cd and Cu than individuals collected in the remaining sampling sites. Lead bioaccumulation levels did not show any spatial pattern. Mussels from the Basque coast presented Pb concentrations ranging between 0.02 mg kg^{-1} dw (in mussels from Hondarribia in autumn) to 0.43 mg kg^{-1} dw (in mussels from Getxo in autumn). Accordingly, despite the lack of a clear spatial gradient, mussels from Pasaia presented the lowest Hg values at all seasons, whereas the highest levels were found in mussels from Getxo in autumn. Mussels from all the sampling sites revealed very variable Zn levels during the study period showing values ranging from 105.99 mg kg^{-1} dw (in mussels from Hondarribia in autumn) to 458.23 mg kg^{-1} dw (in mussels from Getxo in winter). Seasonal variations were not very marked at any sampling site.

Table 2.3. Seasonal metal bioaccumulation levels (mg kg^{-1} dw) in mussels collected in the five sampling sites of the Basque coast. n.d.: no data.

Sampling site	Season	Cd	Cu	Pb	Hg	Zn
Getxo	Winter	1.09	7.54	0.10	0.10	211.35
Gorliz		0.55	5.35	0.16	0.15	357.47
Mundaka		0.49	4.51	0.10	0.11	310.25
Pasaia		0.46	14.39	0.16	0.05	233.05
Hondarribia		0.42	7.95	0.13	0.11	240.22
Getxo	Spring	6.39	12.66	0.17	0.32	458.23
Gorliz		0.96	6.46	0.05	0.26	296.03
Mundaka		1.00	6.34	0.08	0.24	270.99
Pasaia		1.36	22.94	0.10	0.13	352.65
Hondarribia		0.84	9.10	0.25	0.24	284.59
Getxo	Summer	2.15	9.43	0.09	0.20	n.d.
Gorliz		0.54	5.82	0.08	0.17	264.35
Mundaka		0.56	4.63	0.07	0.17	199.47
Pasaia		0.43	19.60	0.09	0.03	286
Hondarribia		0.51	8.08	0.18	0.06	346
Getxo	Autumn	0.54	8.07	0.43	1.95	211.87
Gorliz		0.44	5.12	0.10	0.16	218.60
Mundaka		0.31	n.d.	0.41	0.23	363.82
Pasaia		1.31	2.15	0.14	0.09	362.60
Hondarribia		0.10	1.95	0.02	0.02	105.99

3.3. Digestive gland histopathology

The histological description of the digestive gland was carried out taking into account all the sampling sites and seasons together in order to give a general overview of the main histopathological alterations recorded in this organ. Digestive gland diverticula are organised into clusters of alveolo-tubules linked with the stomach by ducts. These alveolar-tubule units are constituted by a single epithelium, which contains digestive and basophilic cells. The intertubular tissue, *i.e.* connective tissue, was mainly characterised by the presence of haemocytes, mostly granulocytes (Figure 2.2A). Atrophy of digestive tubules was the most significant alteration, with prevalence values between 50 and 100%. This histopathological feature consisted of a reduction in the thickness of epithelia accompanied by the enlargement of the digestive tubule lumen (Figure 2.2B). The highest prevalence of this alteration was recorded in mussels collected in autumn, especially in Getxo, Pasaia and Hondarribia where all of the mussels presented atrophied digestive tubules. In some cases, severe atrophy of digestive tubules was associated with necrosis (Figure 2.2C), that presented noticeable eosinophilia. Necrosis was also observed in intertubular tissue, although its prevalence was lower (digestive tubule necrosis: 10 – 75%; intertubular necrosis: 10 – 60%). Again, necrosis was more frequent in autumn-collected mussels. Intertubular fibrosis was restricted to small foci (0 – 47%) and was normally associated to moderate or severe necrosis (Figure 2.2C). Conversely, this alteration was more common in spring. Fibrosis was developed into a fibroma (benign tumour) only in three individuals within all samples. Malignancies were very rare, whereby only one haemocytic neoplasia was found in one mussel from Pasaia (Figure 2.2D). Inflammation-related alterations were common in the digestive gland and were typically associated with deteriorated tissue or, to a lesser extent, infections. Within these alterations, haemocytic infiltration was the most common (Figure 2.2F). Infiltrating haemocytes comprised hyalinocytes, granulocytes and “brown cells”, the latter characterised by containing aggregates of yellowish-brown lipofuscin-like substances (Figure 2.2E). Overall, these alterations were recurrently recorded and presented values ranging from 15 to 80% for haemocytic infiltration (Figure 2.2F), from 5 to 75% for brown cells and from 5 to 72% for

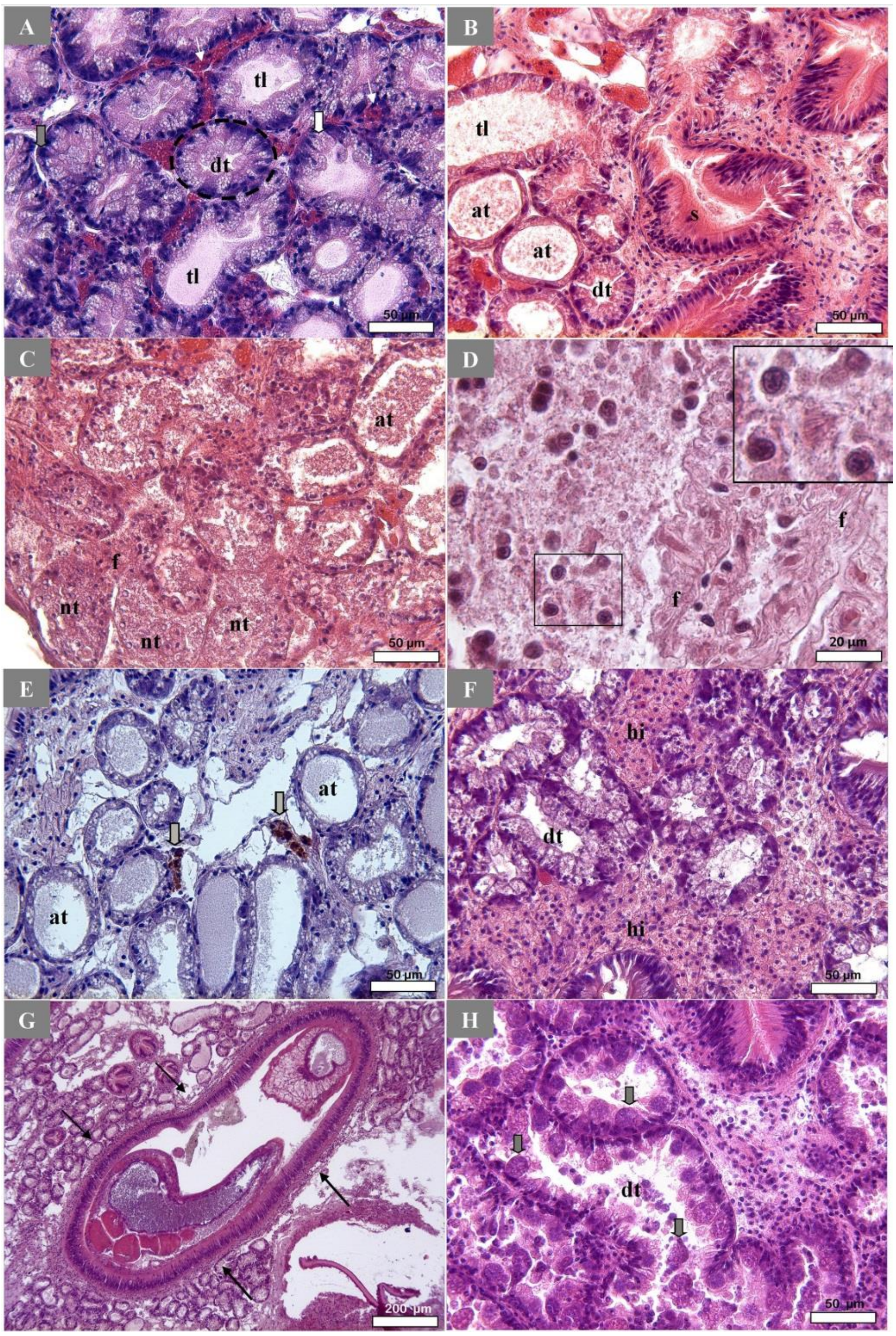

Figure 2.2. Micrographs of representative histopathological lesions observed in the digestive gland of mussels collected from five sampling sites along the Basque coast stained with H&E. (*A*) Normal structure of a digestive gland of a mussel with digestive tubules (dt) being constituted by a single layer of digestive (white wide arrow) and basophilic (grey wide arrow) cells surrounding a narrow or occluded tubular lumen (tl) and connective tissue with several granulocytes (narrow white arrows). (*B*) Atrophied digestive tubules (at), showing a reduction of epithelial cells layer and an enlargement of lumen (tl), together with normal tubules (dt) close to stomach branching duct (s), in a mussel from Getxo. (*C*) Necrotic tubules (nt) together with slight fibrosis (f) and atrophied tubules (at) of a mussel from Hondarribia. (*D*) Haemocytic neoplasia, with a degraded area with anaplastic cells (rectangle) surrounded by fibrosis (f), found in an individual from Pasaia. (*E*) Inflammatory response of a mussel showing brown cells (wide arrows) associated to lipofuscin-like pigments surrounded by atrophied tubules (at). (*F*) A haemocytic infiltration (hi) around digestive tubules (dt) of a mussel from Pasaia. (*G*) *Mytilicola intestinalis* (arrows) in an intestine of a mussel from Mundaka. (*H*) *Marteilia* sp. (wide arrows) in epithelial cells of digestive tubules (dt) of a mussel.

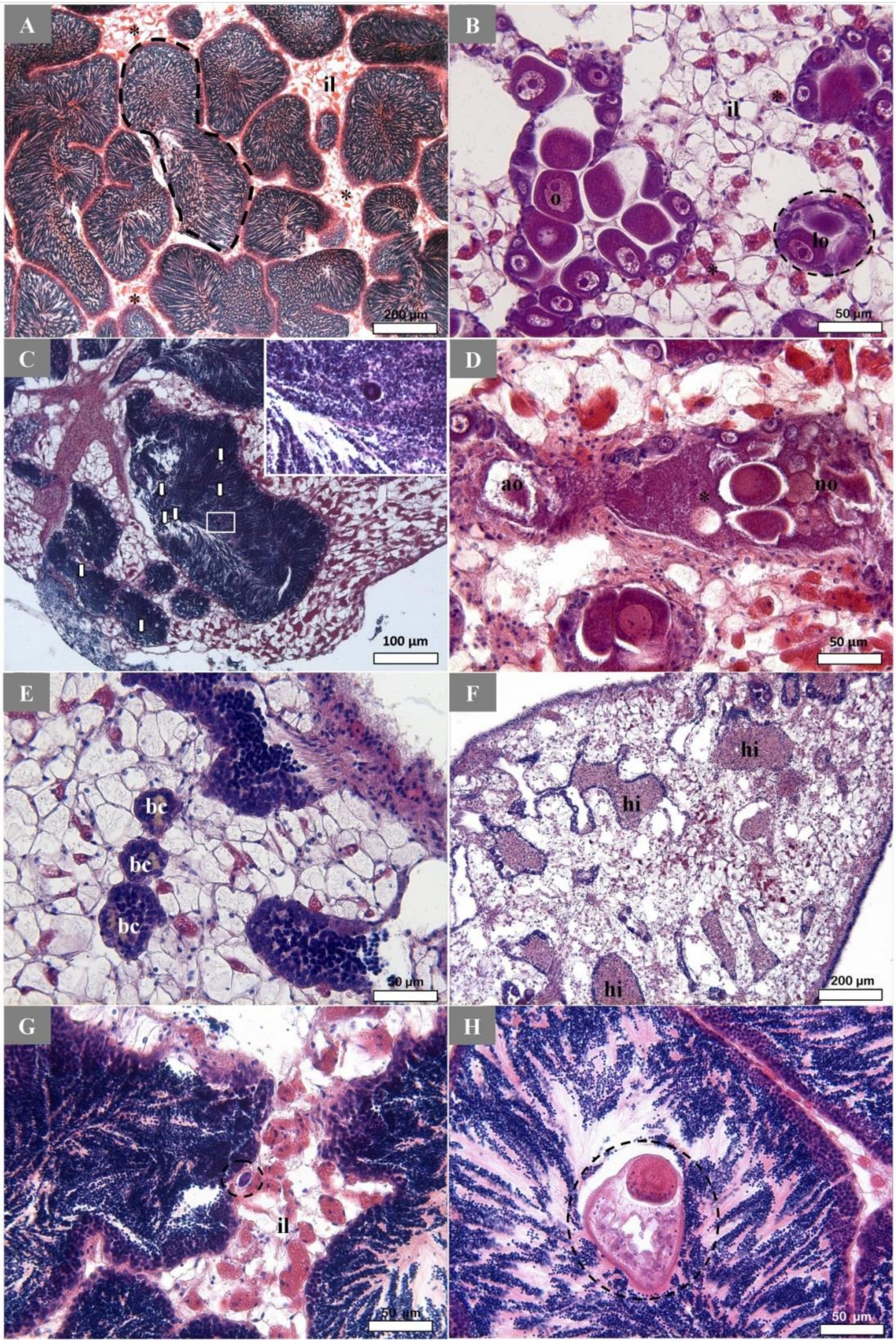

Figure 2.3. Representative micrographs of recorded histopathological lesions in the gonad of mussels collected from five sampling sites along the Basque coast stained with H&E. (*A*) Normal male gonad in a mid-spermatogenic stage showing germinal lobules (dotted line) with plenty of primary and secondary spermatocytes and a few adipogranular cells (*) within interlobular tissue (il). (*B*) Normal female gonad presenting germinal lobules (lo) with oocytes (o) with adipogranular cells (*) throughout interlobular tissue (il). (*C*) Male germinal lobules with oocytes (arrows and rectangle) indicating a slight case of hermaphroditism of an individual from Hondarribia. (*D*) Female gonad tissue with atretic (ao) and necrotic (no) oocytes. (*E*) Male gonad tissue with brown cells (bc) associated to lipofuscin-like pigments within lobular area. (*F*) Female tissue indicating haemocytic infiltrations (hi) in lobular areas. (*G*) *Nematopsis* sp. (circled) in interlobular tissue (il) of a male from Gorliz. (*H*) *Turbellaria* sp. (dashed circled) in a germinal lobule of a male mussel from Gorliz.

lipofuscin-like aggregates. Brown cells were recorded in digestive tubule epithelium and in intertubular tissue, but especially in the latter. Granulocytomas were infrequent, representing only 6% of mussels collected from Pasaia during the whole year except in spring. This alteration was sometimes related to the presence of infections. Regarding parasites, the abundance and diversity was higher in the digestive gland than in the gonad and the lowest prevalence of parasites was attained in spring and summer. Mussels from Gorliz and Mundaka (low impacted sites) were the most parasitised animals (*Nematopsis* sp. 70 – 90%; *M. intestinalis* 10 – 25%), while mussels from Pasaia and Getxo (highly industrialised) were the least (*Nematopsis* sp. 0 – 20%; *M. intestinalis* 0 – 10%). *Nematopsis* sp. was the most common parasite in the digestive gland (up to 94%) and it was observed in intertubular tissue of the digestive gland, typically in foci adjacent to the stomach, and often associated with haemocytic infiltration. This parasite was observed mainly in mussels from Gorliz and Mundaka. Infection by *M. intestinalis* (Figure 2.2G) was less frequent than *Nematopsis* sp., reaching a prevalence of 50% in Hondarribia. *Marteilia* sp. situated in epithelial cells of digestive tubules was the most common parasite in mussels from Mundaka and Hondarribia collected in the summer. In the most extreme cases, this protozoan was also found in the lumen of digestive tubules (Figure 2.2H). Digestive gland copepods were registered only in one or two mussels per site, while Metacercariae cysts and nematodes were detected infrequently.

3.4. Gonad development and histopathology

For the description of gonad sections, all the sampling sites and seasons were considered together in order to characterise the main histopathological lesions of this organ. The gonadal cycle of mussels showed gametogenic stages from winter to early spring; followed by a spawning peak in late spring and summer. In autumn, resting stages were observed, characterised by the reabsorption phase and evidence of the onset of a new gametogenesis cycle. No apparent gamete maturation differences were found between mussels from different sampling sites and genders. The gonad tissue was composed of germinal follicles and connective tissue showing numerous adipogranular cells, recognised by strong eosinophilia and granular appearance (Figures 2.3A and 2.3 B). Interlobular alterations were more pronounced than intralobular lesions. Haemocytic infiltration was common within germinal follicles (0 – 41%) and in interlobular tissue (17 – 100%). This alteration, within germinal lobules, was more common in females (Figure 2.3F). Still, its severity and dissemination were lower than in interlobular tissue. Granulocytomas were also recorded, especially in mussels from Getxo and Pasaia, although with low prevalence (15 – 25%). Undifferentiated mussels yielded the highest prevalence of this lesion. Granulocytomas were more common in gonads than in digestive glands, albeit seemingly unrelated to infections. Fibrosis (0 – 42%) was observed within connective tissue but fibromas were not found in the gonad of any mussel. Oocyte atresia was observed in germinal lobules at all development stages (Figure 2.3D). Female mussels showed higher oocyte atresia values in advanced maturation stages (0 – 66%) than in early stages (0 – 40%). Necrotic oocytes (exhibiting severely disrupted cellular membrane with subsequent plasmalemma rupture and content leakage) were also recorded in follicles with signs of atresia. Only a single case of hermaphroditism with mild dissemination was observed in a male mussel collected from Hondarribia in autumn (Figure 2.3C). Inflammation-related alterations, such as infiltration of brown cells, were also evident in intralobular tissue (0 – 66%) (Figure 3.2E) and in interlobular tissue (0 – 57%) in both genders. Inflammation was more frequent in males than in females, especially in autumn and winter. Apoptosis was occasionally found in intralobular tissue. With regard to parasites, *Nematopsis* sp. was the most common parasite in the gonad, showing a prevalence of 50% (Figure 2.3G). *Turbellaria* sp. was recorded infrequently, especially in the seminiferous tubules of male mussels from Mundaka (Figure 2.3H).

3.5. Histopathological indices in the digestive gland and gonad

Thirteen alterations for the digestive gland and ten for the gonad were considered for the estimation of histopathological indices in mussels (Table 2.1). Histopathological indices were calculated for the digestive gland and the gonad in order to assess the degree of affection of mussels from each sampling site, taking into account the effect of season and parasitosis (Figures 2.4 and 2.5).

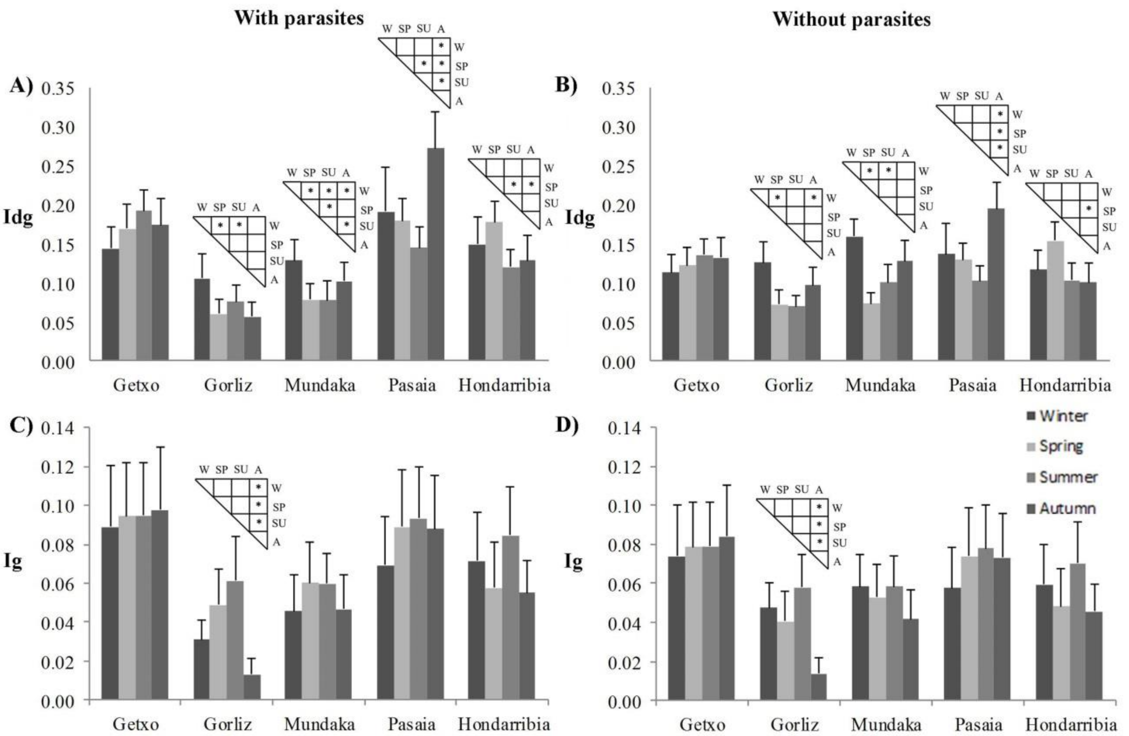

Figure 2.4. Mean histopathological indices determined for each season in the digestive gland (*A*, with parasites; *B*, without parasites) and gonad (*C*, without parasites; *D*, with parasites) of *M. galloprovincialis*. Error bars indicate 95% confidence intervals. The asterisks in the upper triangular matrix indicate significant differences between seasons (Mann-Whitney U test, p <0.05). Idg: histopathological indices of the digestive gland; Ig: histopathological indices of the gonad.

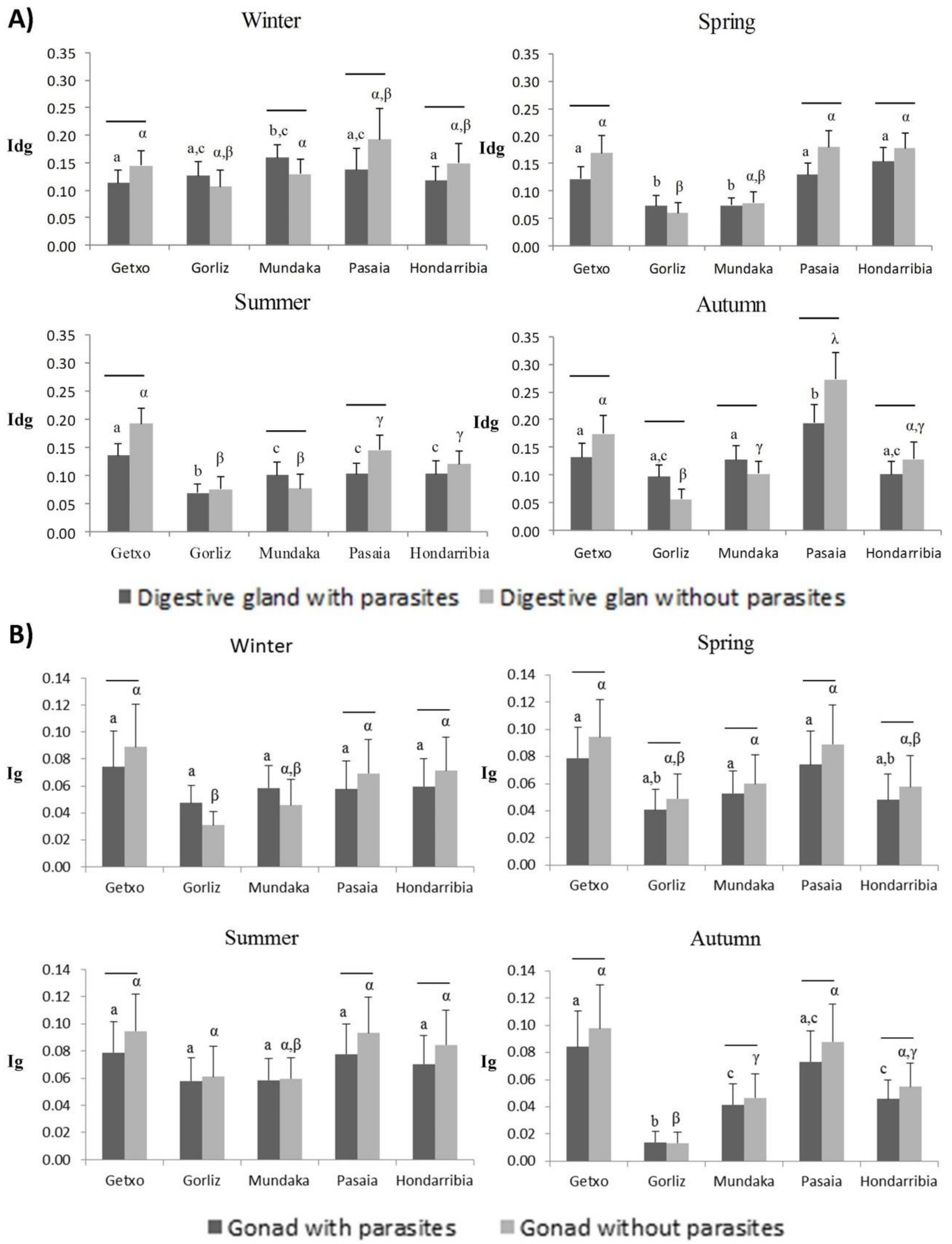

Figure 2.5. Mean histopathological indices determined in the (*A*) digestive gland and (*B*) gonad of *M. galloprovincialis* with and without parasites for each season. Error bars indicate 95% confidence intervals. Different letterings (Latin for indices with parasites whilst Greek for indices without parasites) indicate significant differences between sampling sites (Mann-Whitney U test, $p < 0.05$). Top bars show significant differences between histopathological calculated with and without parasites (Wilcoxon matched pairs test, $p < 0.05$). Idg: histopathological indices of the digestive gland; Ig: histopathological indices of the gonad.

In general, mussels from Getxo and Pasaia showed the highest histopathological indices in both organs, while mussels from Gorliz and Mundaka presented the lowest values. There were significant seasonal fluctuations in the histopathological indices of the digestive gland estimated with and without parasites at all sampling sites, except in Getxo, where no significant seasonal differences were obtained (Figure 2.4). However, these seasonal variations did not follow a repetitive pattern at the different sampling sites. In contrast, histopathological indices calculated in the gonad remained unchanged within the year in mussels from all sites except those from Gorliz, which revealed the lowest values in autumn. The same seasonal pattern was obtained when the gonad histopathological index was calculated with and without parasites (Figure 2.4).

Table 2.4. Histopathological indices of the digestive gland (mean ± 95% confidence intervals) per reaction pattern for each sampling site and season. Different letters indicate significant seasonal differences per reaction pattern and sampling site (Mann-Whitney U test, p < 0.05). n.o.: not observed.

Sampling site	Season	Tubule alterations	Intertubular alterations	Parasitosis
Getxo	Winter	0.27 ± 0.6	0.08 ± 0.03	0.04 ± 0.03
	Spring	0.26 ± 0.05	0.10 ± 0.03	0.01 ± 0.01
	Summer	0.31 ± 0.04	0.10 ± 0.03	0.02 ± 0.02
	Autumn	0.28 ± 0.05	0.10 ± 0.03	0.03 ± 0.02
Gorliz	Winter	0.16 ± 0.04 [a]	0.07 ± 0.03 [a]	0.17 ± 0.06 [a]
	Spring	0.07 ± 0.03 [b]	0.05 ± 0.02 [a]	0.10 ± 0.04 [b]
	Summer	0.10 ± 0.04 [b]	0.06 ± 0.03 [a]	0.05 ± 0.03 [b]
	Autumn	0.11 ± 0.04 [a]	0.02 ± 0.01 [b]	0.19 ± 0.07 [c]
Mundaka	Winter	0.25 ± 0.06 [a]	0.04 ± 0.02	0.23 ± 0.05 [a]
	Spring	0.10 ± 0.04 [b]	0.06 ± 0.02	0.06 ± 0.04 [b]
	Summer	0.10 ± 0.04 [b]	0.06 ± 0.03	0.16 ± 0.05 [c]
	Autumn	0.16 ± 0.04 [c]	0.06 ± 0.03	0.19 ± 0.05 [a,c]
Pasaia	Winter	0.31 ± 0.07 [a]	0.11 ± 0.05 [a]	0.01 ± 0.01
	Spring	0.32 ± 0.05 [a]	0.03 ± 0.03 [b]	0.01 ± 0.01
	Summer	0.23 ± 0.04 [b]	0.08 ± 0.03 [a,b]	n.o.
	Autumn	0.39 ± 0.07 [c]	0.19 ± 0.05 [c]	0.01 ± 0.01
Hondarribia	Winter	0.23 ± 0.03	0.09 ± 0.03	0.04 ± 0.02 [a]
	Spring	0.23 ± 0.04	0.11 ± 0.04	0.01 ± 0.02 [b]
	Summer	0.17 ± 0.04	0.08 ± 0.03	0.06 ± 0.04 [a,b]
	Autumn	0.20 ± 0.04	0.08 ± 0.03	0.03 ± 0.03 [a,b]

Besides, histopathological indices in the digestive gland presented significantly higher values than in the gonad in all sampling sites and seasons (note the different scales in Figures 2.4 and 2.5).

Table 2.5. Histopathological indices of the gonad (mean ± 95% confidence intervals) per reaction pattern for each sampling site and season. Different letters indicate significant seasonal differences per reaction pattern and sampling site (Mann-Whitney U test, $p < 0.05$). n.o.: not observed.

Sampling site	Season	Lobular alterations	Interlobular alterations	Parasitosis
Getxo	Winter	0.08 ± 0.04	0.10 ± 0.05	n.o.
	Spring	0.13 ± 0.03	0.07 ± 0.03	n.o.
	Summer	0.14 ± 0.04	0.06 ± 0.05	n.o.
	Autumn	0.10 ± 0.03	0.10 ± 0.06	n.o.
Gorliz	Winter	0.02 ± 0.02 ^{a}	0.04 ± 0.02 ^{a}	0.13 ± 0.09 ^{a}
	Spring	0.02 ± 0.02 ^{a}	0.07 ± 0.03 ^{a}	n.o.
	Summer	0.05 ± 0.02 ^{a}	0.07 ± 0.05 ^{b}	0.04 ± 0.06 ^{a,b}
	Autumn	0.01 ± 0.01 ^{b}	0.01 ± 0.01 ^{a}	0.02 ± 0.03 ^{b}
Mundaka	Winter	0.04 ± 0.02	0.05 ± 0.03	0.12 ± 0.07 ^{a}
	Spring	0.06 ± 0.02	0.06 ± 0.04	0.02 ± 0.03 ^{b}
	Summer	0.06 ± 0.02	0.06 ± 0.03	0.05 ± 0.06 ^{a,b}
	Autumn	0.06 ± 0.02	0.04 ± 0.03	0.02 ± 0.03 ^{b}
Pasaia	Winter	0.06 ± 0.02	0.08 ± 0.04	n.o.
	Spring	0.10 ± 0.02	0.08 ± 0.04	n.o.
	Summer	0.07 ± 0.03	0.11 ± 0.04	n.o.
	Autumn	0.12 ± 0.04	0.06 ± 0.04	n.o.
Hondarribia	Winter	0.08 ± 0.04	0.07 ± 0.04	n.o.
	Spring	0.06 ± 0.02	0.05 ± 0.04	n.o.
	Summer	0.12 ± 0.04	0.05 ± 0.03	n.o.
	Autumn	0.09 ± 0.03	0.03 ± 0.02	n.o.

The histopathological indices calculated per reaction pattern within a year for the digestive gland and the gonad are summarised in Tables 2.4 and 2.5, respectively. Overall, digestive tubule alterations and lobular alterations for gonads were the reaction patterns with the highest histopathological indices. All reaction patterns demonstrated seasonal fluctuations in both organs but without a repetitive pattern. With regard to parasites, mussels from the highly industrialised sites such as Getxo and Pasaia practically showed absence lack of infections in both organs during the year; conversely mussels from Gorliz and Mundaka, considered as low impacted sites, presented the highest frequencies of infections (Tables 2.4 and 2.5). Thereby, the presence of parasites demonstrated a high influence in histopathological indices, increasing in value in low impacted areas and decreasing in value in highly impacted zones.

The digestive gland histopathological index showed significant differences in the values obtained with and without parasites, especially in mussels from Pasaia and Getxo (Figure 2.5). In highly polluted sites (Getxo and Pasaia) parasitosis interfered during the four seasons of the year, while the effect of parasitosis was not so evident throughout the year (Figure 2.5) in low polluted sites (Gorliz and Mundaka). In the case of gonads, a similar pattern was observed (Figure 2.5).

3.6. Data integration

Two models were designed by discriminant analyses considering all reaction patterns of both target organs and the gonad index, while season and gender were analysed as independent variables (Table 2.6). According to Model 1, autumn was the most discriminant season given by the lowest Wilks' λ for inter-site distinction (Wilks' λ = 0.1740). All parameters were significant within this first model except interlobular alterations and parasitosis in the gonad. In the second model, both independent parameters (season and gender) were combined, whereby female mussels in autumn presented the best model (Wilks' λ = 0.0378) for inter-site comparison. In this case, the gonad index and digestive tubule alterations were the most significant and discriminant variables.

Table 2.6. Summary of discriminant analyses. The significance of each parameter is given by partial Wilks' λ and F-test. In model 1, data from autumn was considered; in model 2, season and sex variables were combined using data of female mussels collected in autumn.

	Parameters	**Model 1** Autumn		**Model 2** Autumn - Female	
		Partial λ	*p*	Partial λ	*p*
	Gonad Index	0.7917	0.0003	0.4004	0.0016
Digestive gland	Tubule alterations	0.7685	0.0001	0.5125	0.0131
	Intertubular alterations	0.8844	0.0274	0.8433	0.5199
	Parasitosis	0.6946	0.0000	0.6162	0.0570
Gonad	Lobular alterations	0.7823	0.0002	0.7148	0.1738
	Interlobular alterations	0.9091	0.0755	0.8223	0.4469
	Parasitosis	0.9785	0.7810	0.7638	0.2767

Regarding chemical and biological data integration, no correlations were found between metal bioaccumulation levels and histopathological indices in the digestive gland and gonad.

4. Discussion

This study developed a histopathological index in mussels to enable the integration of all alterations into a single value in order to facilitate the interpretation of the health status of mussels from different sites collected in four seasons. The development of quantitative histopathological tools is potentially expeditious and highly informative for the monitoring of stressor-induced biological effects under the scopes of the MSFD's Descriptor 8. Nonetheless, the current work also showed that knowledge on biotic and abiotic confounding factors is essential to identify the background response, the moderate response and the significant response that can be objectively pinpointed to populations collected from different sampling areas.

Detailed histopathology of the digestive gland and gonad revealed distinct patterns of lesions in mussels from different sampling sites. Mussels collected from Gorliz yielded the lowest histopathological indices; although this was not reflected in metal bioaccumulation levels and SQG-Qs. These low histopathological indices could be related with the high hydrodynamic conditions owing to its exposure to the open sea (Belzunce et al., 2004a; Orbea et al., 2002). The lowest SQG-Qs were recorded in Mundaka, situated in the Urdaibai Biosphere Reserve, but mussels showed higher histopathological indices in both organs than mussels from Gorliz. This could be explained by the industrial activities (metallurgic and shipyard) and the inefficiency of the sewage treatment plant situated upstream (Puy-Azurmendi et al., 2013). The most deteriorated areas were Getxo and Pasaia, although mussels from Pasaia in general presented significantly higher histopathological indices in the digestive gland than mussels from Getxo. Both sites were subtidal estuaries markedly impacted by anthropogenic activities (*i.e.* commercial harbours, leisure ports and diverse industries), but Pasaia due to its narrow mouth to the open sea presented a lower hydrodynamic environment than Getxo (Solaun et al., 2013) which may have contributed, at least partly, to the higher tissue damage. Although in recent years, the improvement of both estuaries was appreciable (Borja et al., 2009; Solaun et al., 2013), histopathological indices were significantly higher than those recorded in mussels from Gorliz indicating that high contaminant levels still persist, as inferred from the levels of pollutants in sediments. Hondarribia could be regarded as a moderately impacted site because it presented intermediate histopathological indices, but SQG-Qs were as low as those of Gorliz and Mundaka. This estuary was characterised by high hydrodynamics and a sandy environment, which contributed to a low SQG-Qs. Overall, Cu, Hg, Pb, Zn, PCBs and low molecular weight PAHs were contaminants of concern due to their high toxicological significance along the Basque coast. Nevertheless, the high content of Cu, Pb and Zn in sediments may derive from a natural enrichment of the Basque basins (Belzunce et al, 2004b). In highly impacted areas, such as Getxo and Pasaia, sediment pollution levels were consistent with the histopathological indices. In moderate or low impacted sites, however, this relation was less obvious, suggesting that contamination levels did not cause significant deleterious effects on mussels' health status. It is worth noting that mussels reflect the pollution of the water column rather than of sediments, which indicates that histopathological alterations might not be directly related to sediment pollution levels, especially at sites with low SQG-Qs. Accordingly, no significant correlations were obtained between metal bioaccumulation levels and histopathological indices recorded in the digestive gland and gonad of mussels. This could be partly explained by the fact that other factors might be the causing agents of such tissue damage or by the fact that metal concentrations were not high enough to produce adverse effects. Furthermore, only mussels from Getxo and Pasaia exceeded the upper limit of background range proposed for Cd, Cu and Hg (only mussels from Getxo) as the transition point between "high" status and "good" status according to the WFD (Solaun et al., 2013).

Regarding histopathological traits, the digestive gland of molluscs is the main organ for metabolic regulation, mechanisms of immune defence and homeostatic regulation, together with processes of detoxification of xenobiotic compounds (Moore and Allen, 2002), thus it is considered as a main target organ in toxicological studies (Bignell et al., 2008, 2011; Marigómez et al., 2013). Histopathological alterations of the digestive gland, such as digestive tubule atrophy and necrosis, were the most significant lesions. Hence, in mussels treated with Cd digestive tubule atrophy was shown as a key step in initial cellular necrosis (Da Ros et al., 1995). Inflammatory responses were also highly prevalent; within which haemocytic infiltration was the most frequent alteration. This latter lesion is regarded as an important biomarker of inflammatory response in bivalves; furthermore, some works link this alteration to severe lesions caused by neoplasia, parasitosis or xenobiotics (Sheir and Handy, 2010; Villalba et al., 2001). Also, although a common alteration, often related to age, feeding and other factors, some authors found that relatively large amounts of lipofuscin-like pigments, as those comparatively observed here in animals from highly polluted sites, may also indicate deteriorated tissue (Carballal et al., 1997). Nonetheless, the deployment of lipofuscin-containing cells as an ecotoxicological biomarker is still prone to debate and further research. A further two closely related lesions were promptly observed: granulocytoma, which reflects symptoms of long-term exposure to contaminants (Lowe and Moore, 1979), and fibrosis that was often related with necrotic areas. Malignancy, considered as fatal neoplasm to several bivalve species and proposed as an indicator of environmental quality in aquatic organisms (Romalde et al., 2011; Sound et al., 2011), was observed in a unique specimen, compromising its value as a biomarker under the present circumstances of assessment and study area. As regards parasites, an important presence of pathogens was recorded in this organ, especially *Nematopsis* sp. and *Mytilicola intestinalis*. The effect of infections in histopathological traits has been studied considerably showing adverse effects (Pérez Camacho et al., 1997). However, parasites in bivalves may positively or negatively alter the resistance of an organism to a chemical agent depending on the severity of infection and chemical exposure (Bignell et al., 2008; Minguez et al., 2012; Morley, 2010).

On the other hand, gonads were also used as a target organ in this study due to their fundamental function in hormone production, gamete development and progress of upcoming generations (Blazer et al., 2002; Fernández-Reiriz et al., 2007). Furthermore, the gonad development analysis also plays an essential role in environmental and physiological assessment of bivalve populations, being essential data included in the Mussel Watch Project (Hillman, 1993). In this study, the gonadal cycle of mussels followed the typical pattern for the Basque Coast populations (Garmendia et al., 2010; Múgica et al., 2015). In spring, mussels were in the spawning stage with follicles full of ripe gametes, while in summer they were in advanced spawning stage with half empty follicles. In autumn, mussels presented gametes in resting stages with a considerable percentage of mussels entering a new reproductive cycle whereas in winter, mussels were in advanced gametogenesis.

Interlobular alterations were the most prevalent lesions, especially haemocytic infiltration, followed by brown cells, to a lesser extent. These inflammatory changes were also present in germinal lobules, manifesting the highest prevalence in autumn. In this season, mussels showed gametes mainly at resting stage when energy storage is exhausted. Thus, histopathological alterations to pollutant exposure should be described carefully, since it could be a natural reabsorption stage of this organ (Brenner et al., 2014; Carballal et al., 1998; Garmendia et al., 2010). Similarly, fibrosis showed the highest prevalence in autumn, which may be related to physiological changes (Ruiz et al., 2011). Although oocyte atresia was more frequent within advanced maturation stages in this research, Ortiz-Zarragoitia and Cajaraville (2010) indicated that high oocyte atresia prevalence values recorded in winter in mussels from the Urdaibai Biosphere Reserve was associated with a lack of favourable conditions for spawning after gamete ripening. In many studies, suppressed gamete development, inhibition of gonadal follicle development and enhancement of oocyte atresia in mussels were associated to pollutant exposure (Ortiz-Zarragoitia and Cajaraville, 2010; Puy-Azurmendi et al., 2010). Hermaphroditism was also shown in one mussel from Hondarribia collected in autumn, suggesting signs of exposure to endocrine disrupting chemicals but more data is needed to confirm this hypothesis.

One of the main drawbacks of environmental pollution biomonitoring is the presence of potential confounding factors that may lead to a misinterpretation of the toxicological findings (Costa et al., 2013; Garmendia et al., 2010). This study revealed parasitosis as a significant and potential confounding factor. Parasites appeared abundantly in both organs of mussels with ‘good’ health status, such as those from Gorliz and Mundaka. Conversely, Pasaia and Getxo, which were considered to be the most polluted areas, presented mussels with almost no parasites. Thus, it seems that in low impacted sites parasites overestimated the real histological damage, while in strongly polluted sites the real histological harm was underestimated. This fact revealed that histopathological indices considering parasites can become less sensitive to inter-site comparisons, since they may mask the real health status of mussels (Costa et al., 2013; Minguez et al., 2011, 2012). However, the estimation of both histopathological indices, with and without parasites, should be carried out in order to identify those histopathological alterations related to infections. Hence, this predisposition may reveal that parasites can be proposed as a susceptibility biomarker candidate (Costa et al., 2013).

Regarding season-related effects, mussels, like all aquatic poikilotherm and particularly in temperate climate zones, such as the Basque coast, are characterised for having pronounced seasonal cycles related to their physiology and reproduction (Bignell et al., 2008; Brenner et al., 2014; Garmendia et al., 2010). Thus, seasonality is a critical factor that needs to be taken into consideration in pollution assessment (Bocchetti and Regoli, 2006) using the biomarkers approach (Rickwood and Galloway, 2004; Schmidt et al., 2013). In this study, significant seasonal fluctuations in histopathological indices of the digestive gland were observed in almost all the Basque estuaries, except in Getxo, where no variations were recorded. This may suggest that mussels from Getxo have a slow or non-recoverable response to stressful environmental conditions (Brown et al., 1992). In previous studies, seasonal changes in various biomarkers were observed in mussels from Mundaka, but they were largely attenuated in mussels from Getxo, which is more polluted than Mundaka (Cearreta et al., 2004; Múgica, 2014; Orbea et al., 2002; Orbea and Cajaraville, 2006) and richer in organic nutrients that are subject to less seasonal and inter-annual variations (Díaz et al., 2003; Uriarte and Villate, 2004). In the gonad, however, no significant seasonal variations were recorded in histopathological lesions. Therefore, as in Garmendia et al. (2010), this study highlighted the importance of seasonality as a confounding factor in histopathological interpretation and consequently biomonitoring during the same season is recommended. In view of the above, autumn, in particular, was the most suitable season for inter-site histopathological avoiding the natural physiological responses of advance maturation stages. This was consistent with previous studies using mussels as sentinels in the Basque coast in which autumn was recommended as the most appropriate season for sampling (Besada et al., 2008; Garmendia, et al., 2010; Solaun et al., 2013). Nevertheless, mussels can exhibit significant biological variability within a same population with regard to reproductive condition, which may be related with genetic performance and resilience, rendering mandatory a deep knowledge of the species and population to be analysed as bioindicator (Bignell et al., 2008; Secor et al., 2001).

5. Conclusions

This study contributed to the knowledge of histopathology in two target organs (digestive gland and gonad) of the mussel, the sentinel species most widely used in Mussel-Watch Programs. The integration of multiple histopathological traits into a single index permitted a more sensitive discrimination of different sites and provided a ranking of sampling sites in accordance with environmental variables, such as sediment characteristics, hydrodynamics and source of contamination, and Cd and Cu bioaccumulation levels. Mussels from the most impacted sites (Getxo and Pasaia) endured the most significant deleterious effects. However, the findings indicate that parasitosis and seasonal variations act as confounding factors and as such, they should be accounted for in environmental monitoring research. Parasitosis masked the real tissue damage, underestimating the histopathological index in high polluted sites and overestimating it in low impacted sites. Seasonal variations demonstrated that inter-site differences were more pronounced in autumn, when natural physiological responses of advanced maturation stages do not interfere in the histological response. On the other hand, it has become clear that the choice of the target organ is paramount. As such, regardless of the importance of the gonads for the population fitness, the digestive gland, *i.e.*, the organ analogous to the vertebrate liver with respect to toxicant accumulation and metabolism yielded more conclusive histopathological findings if cause-effect relationships are to be sought.

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Chapter 3

Multi-organ histopathology in gobies for estuarine environmental risk assessment: A case study in the Ibaizabal estuary

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Summary

Multi-organ (liver, gills, kidney and spleen) histopathology in gobies (*Pomatoschistus* spp.) together with metal bioaccumulation and sediment contamination levels were studied during three years (2011 – 2013) for estuarine environmental risk assessment in the Ibaizabal estuary. Results indicate that sediments were moderately-strongly impacted by metals and organic compounds, suggesting that adverse biological effects could be likely. Similar metal bioaccumulation levels and multi-organ histopathological indices were detected in gobies collected along the estuary, indicating a similar affection degree. Accordingly, both metal bioaccumulation levels and histopathological indices were lower in gobies from the most recent campaign reflecting a lower impact on fish health status. Liver, gills and kidney reached higher histopathological damage than spleen. Fat vacuolation of hepatocytes, lamellar fusion and melanomacrophage centers were the most prevalent hepatic, branchial and renal alterations. These histopathological changes may indicate exposure to non-specific toxicants, although the influence of other unknown environmental factors should not be excluded. No severe pathological traits were registered in gonads, suggesting undisturbed reproductive status. In conclusion, the use of multi-organ histopathology in gobies in combination with metal bioaccumulation and sediment contamination levels, contribute to a better understanding of sub-lethal effects and a more accurate environmental risk assessment in the Ibaizabal estuary.

1. Introduction

Estuaries are major sinks of potentially hazardous chemicals derived from human activities. Most of these substances are highly persistent and may provoke adverse effects on estuarine organisms, with severe consequences for ecosystem health, local economies and human health (Borja et al., 2008; Chapman et al., 2013; Gredilla et al., 2013). Hence, the monitoring and protection of estuarine ecosystems has long been regarded as a priority for environmental protection agencies and stakeholders worldwide (see for instance Monteiro et al., 2006; Stentiford et al., 2003). Within the estuarine environment, sediments constitute a dynamic and essential component, at least due to their provision of habitat and food resources to benthic and demersal species (Apitz, 2012). However, aquatic sediments, especially those of confined coastal waterbodies, also tend to accumulate many pollutants, since many substances tend to be adsorbed to particular matter or to become trapped in pore water (Martínez-Lladó et al., 2007).

Traditionally, sediment contamination risk has been determined by assessing the bulk chemical content of each toxicant with the subsequent comparison with background or reference values (Belzunce et al., 2004; Rodríguez et al., 2006). Due to the little insight on the true ecological impact retrieved from this approach, more integrated assessments are recommended, implying the analysis of different LOEs in order to link sediment contamination to adverse biological effects (Montero et al., 2013). In this respect, Chapman et al. (1997) proposed the Sediment Quality Triad, which integrates contamination levels, toxicity testing and benthos analysis for sediment risk assessment. Later on, this integrated approach has been updated integrating other LOEs (Chapman and Hollert, 2006; Chapman et al., 2013). Among these LOEs, bottom fish histopathology has been recommended and effectively employed in many biomonitoring programmes (*e.g.* Costa et al., 2009; Fricke et al., 2012; Lang et al., 2006; Schultz et al., 2013; Stentiford et al., 2003), due to its ability to disclose the true health status of animals (Chapman and Hollert, 2006). Liver, gills, kidney and spleen, together with gonad as supporting organ, have already been assessed histopathologically as target organs in field or laboratory works (Costa et al., 2010; Monteiro et al., 2006; Schultz et al., 2013).

Teleosts have been widely used in ecotoxicological studies due to their ecological relevance, availability and ability to act as surrogates for higher vertebrates (Costa et al., 2013). The gobies, *Pomatoschistus* spp., are ubiquitous and abundant benthic species in most European estuaries and near shore waters, and as such, they are susceptible to sustain contamination induced injury (Dolbeth et al., 2007; Fonseca et al., 2014; Martinho et al., 2006; Stentiford et al., 2003). Gobies are among the most abundant fish species inhabiting the Ibaizabal estuary (Revilla et al., 2014), which makes them suitable sentinel fishes for estuarine sediment monitoring.

The Ibaizabal estuary has been highly impacted for many decades by several human activities such as discharges of urban effluents, mineral sluicing and industrial wastes (Belzunce et al., 2004; Cearreta et al., 2000). Consequently, this estuarine system reached a very bad status showing low oxygen levels, high contaminant contents and disappearance of fauna in several areas (Borja et al., 2006). However, the implementation of modern sewerage systems together with the decline of industrial activity have led in the last years to a gradual improvement of the environmental health status of the system, both in terms of the physico-chemical components and the biological communities (Borja et al., 2010). Nevertheless, although recent studies on sediment toxicity tests using sea urchins and amphipods indicate lack of acute toxicity, sediment contaminants levels are still relevant (Montero et al., 2013). Therefore, in order to contribute to a better assessment of estuarine environmental health status, evaluation of sub-lethal effects (measured in terms of multi-organ histopathology) in gobies together with metal bioaccumulation and sediment contamination levels were studied in a three year survey carried out along the Ibaizabal estuary. Hence, the specific aims of the present study are (*1*) to provide a multi-organ (liver, gills, kidney and spleen) histopathological assessment in gobies and (*2*) to evaluate the use of the multi-organ histopathology in combination with metal bioaccumulation and sediment contamination levels for environmental risk assessment in the Ibaizabal estuary.

2. Material and methods

2.1. Sampling area and sample collection

Sampling campaigns were carried out in the Ibaizabal estuary in autumn with the "Ortze" oceanographic vessel. Sediments were collected from 2009 to 2013 by a Day grab at four sampling sites (a total of 20 sediment samples) for sediment characterisation and contaminant determination (Figure 3.1). Similarly, gobies (*Pomatoschistus* spp.) were collected from 2011 to 2013 by trawling at four transects situated upstream (Olabeaga and Rontegi) and downstream (Lamiako and Inner Abra) the sewage treatment plant (Figure 3.1). Water depth in these areas was between 8 and 15 m. Three hauls of 10 - 15 min were carried out at each sampling transect and survey. No fish were obtained at Lamiako on the 2011 campaign. Approximately 30 individuals per haul were fixed in 10% neutral buffered formalin for 24h at 4ºC for histological examination. Another set of 30 individuals per haul was pooled and stored at -20ºC for metal bioaccumulation analyses.

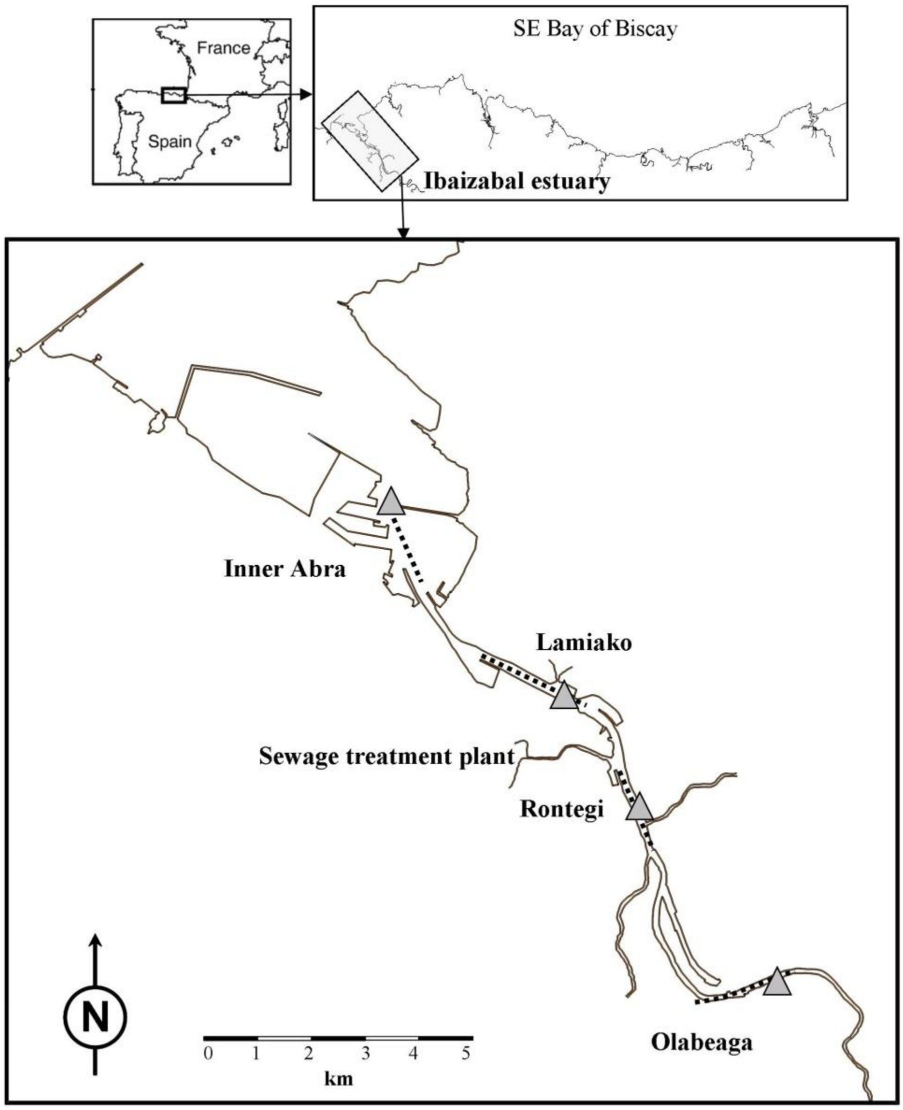

Figure 3.1. Map of the Ibaizabal estuary, showing the four trawling transects (dotted line), the sewage treatment plant and sediment sampling sites (triangles).

2.2. Sediment characterisation and contamination levels

The granulometric characterisation of sediments, % gravel (> 2 mm), % sand (2 mm - 63 µm) and % mud (< 63 µm), was estimated by two procedures depending on the percentage of fine fraction. Samples with low percentage of fine sediment (< 10%) were determined by dry sieving according to Folk (1974); while samples with high content of fine sediments (> 10%) were determined by LDPSA; due to the underestimation of the finest fraction, mud content was transformed following the method proposed by Rodríguez and Uriarte (2009).

Metal (Cd, Cr, Cu, Hg, Ni, Pb and Zn) contents were measured in triplicate in acid extracts from the fine fraction of the sediments (< 63 µm). 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 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 vapor method. Analytical accuracy was checked by the PACS-2 reference material (NRC) and the measured values were found to be within the certified range. Recoveries for the certified metals were: 92% Cd; 70% Cr; 95% Cu; 95% Hg; 99% Ni; 100% Pb and 95% Zn.

Organic compounds such as PCBs, other organochloride pesticides and PAHs were also determined (see Table 3.1). Sediment samples (5-10 g) were pre-concentrated with a mixture of solvents (pentane:dicloromethane; 50:50) by ASE (200 system DIONEX). Organic extract was purified by 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 GC-MS (Agilent 6890 GC coupled with an Agilent 5973 MSD instrument). Mean recoveries for almost all the certified PAHs were between 80 and 90%. Indeno[1,2,3-cd]pyrene and benzo[ghi]perylene showed lower recovery rates of between 60 and 70%. Mean recoveries, for almost all the certified PCBs were between 85 – 110%, while for all the certified DDTs were between 60 – 90%.

Contaminant levels were contrasted with the published values of the TEL and PEL proposed for metals (Menchaca et al., 2012); PCBs and PAHs (Menchaca et al., 2014); plus DDTs (MacDonald et al., 1996). The contaminants potential to cause adverse biological effects was assessed through the estimation of SQG-Qs (Long and MacDonald, 1998). The sediments were then ranked as proposed by MacDonald et al. (2004): SQG-Q < 0.1 as non-impacted sediments; 0.1 – 1 as moderately impacted and > 1 as strongly impacted.

2.3. Metal bioaccumulation levels

Pools of whole gobies were homogenised and approximately 0.5 g of freeze-dried samples were digested with 10 ml of concentrated nitric acid by a microwave oven (MARS 5 Xpress CEM Corporation Instrument). After digestion, Cd, Cu, Hg, Pb and Zn were analysed by AAS (AAS800 Perkin Elmer). Total Hg was determined by the cold vapor technique and the rest of the metals by graphite furnace, equipped with a Zeeman background correction device. Certified reference material DORM-2 (Dogfish muscle) from NRC was used in order to validate the analytical procedure. The mean recoveries were in the range of 80 – 90% for all of the metals, throughout the study.

2.4. Biometric parameters

The weight (g) and the total length (cm) of each fish were recorded and then, the condition factor (K) was calculated as [(weight*100)/(lenght^3)].

2.5. Histology and histopathological procedures

Fixed gobies were dehydrated in a graded series of ethanol, cleared and embedded in methacrylate resin (Technovit 7100, Heraeus Kulzer). Sections, 3-5 µm thick, were obtained with a rotary microtome (HM 350S, Microm) and then stained with H&E.

Histological examination of the liver, gills, kidney, spleen and gonad was carried out under a light microscope (BX60, Olympus).

Firstly, the histological alterations of different organs (liver, gills, kidney and spleen) were described and the prevalence (%) was calculated as (number of cases/total cases analysed) ×100. Secondly, the histopathological indices were estimated in all target organs according to Bernet et al. (1999) and adapted by Costa et al. (2009). The histopathological indices considered the relative biological importance (weight) of each alteration and the degree of dissemination (score) of each lesion within the studied organ. The histopathological alterations were classified into four reaction patterns: (1) circulatory disturbances, (2) inflammatory responses, (3) regressive changes (functional loss) and (4) progressive changes (altered function). The global histopathological indices (Ih) were calculated for each individual and organ as detailed below:

$$Ih = \sum_{1}^{j} w_j \, a_{jh}$$

where w_j is the weight of the *j* histopathological trait and a_j the score for the *j* alteration of the *h* individual. The weights were 1 (slight), 2 (moderate) and 3 (severe); while the score ranged from 2 to 6 depending on the dissemination degree of the alteration. The alterations considered for the estimation of histopathological indices of each target organ and their respective biological importance (weight) are illustrated in Table 3.1. The accuracy of histopathological estimation was checked by a series of blind reviews.

Gonads were analysed as well, taking gender, gamete developmental stages, atresia and intersex as main endpoints.

Table 3.1. Histopathological alterations recorded in liver, gills, kidney and spleen of gobies and their respective biological significance (w).

Liver	**w**	**Gills**	**w**	**Kidney**	**w**	**Spleen**	**w**
Circulatory disturbances							
Haemorrhage	1	Haemorrhage	1	Haemorrhage	1	Haemorrhage	1
Hyperaemia	1	Hyperaemia	1	Hyperaemia	1		
Aneurysm	1						
Inflammatory responses							
MMCs	1	MMCs	1	MMCs	1	MMCs	1
Lymphocytic infiltration	2	Lymphocytic infiltration	2				
Regressive changes							
Necrosis	3	Necrosis	3	Renal tubule regression	2		
HNP	2	Epithelial desquamation	1	Necrosis	3		
		Epithelial lifting	1				
Progressive changes							
FV of hepatocytes	1	HP of chloride cells	2	HP of epithelial cells of renal tubule	1		
Spongiosis hepatis	2	HP of goblet cells	2	HT of epithelial cells of renal tubule	2		
CPF of bile ducts	2	HT of goblet cells	1	HV of epithelial cells of renal tubule	2		
		HT of chloride cells	1				
		Lamellar fusion	1				
		Degeneration of goblet cells	2				

Note: HNP, hepatocellular nuclear pleomorphism; FV, fat vacuolation; CPF, concentric periductal fibrosis; MMCs, melanomacrophage centers; HP, hyperplasia; HT, hypertrophy; HV, hydropic vacuolation.

2.6. Statistical analyses

Statistical analyses were carried out with the aid of Statgraphic Plus 5.0. The normality and homoscedasticity of all parameters was tested by the Shapiro-Wilks and Levene test, respectively. The interaction effects of campaign and transect were analysed according to the non-parametric Bennet's bivariate sign test. The non-parametric Kruskal-Wallis followed by the Mann-Whitney U test were employed for inter-site and temporal comparisons. The non-parametric Spearman's rank-order (R) was used to assess correlations between surveyed variables. A significance level of $p < 0.05$ was set for all statistical analyses.

3. Results

3.1. Sediment characterisation and contamination levels

The characterisation, contamination levels and toxicological significance of sediments from the Ibaizabal estuary are shown in Table 3.2. Olabeaga and Lamiako were characterised by sand predominance, while Rontegi and Inner Abra were mainly muddy. However, TOM ranged between 2.4% and 6.6% in sediments from all sampling sites. There were no significant differences in metal content among sampling areas except for Hg, which was significantly higher at Lamiako, that is located downstream the sewage treatment plant. Olabeaga, the inner most transect, presented significantly lower PCB and PAH levels than the rest transects of the estuary; while DDT levels remained similar along the estuary. The intermediate area of the estuary (Lamiako) showed sediments strongly impacted by metals (Table 3.2), while the rest of the estuary presented sediments moderately impacted.

Table 3.2. Median ± standard deviations of granulometry (%), metal (mg kg^{-1} in dw) and organic contaminant content (μg kg^{-1} in dw) with toxicological significance (SQG-Qs) of sediments collected from 2009 to 2013 along the Ibaizabal estuary. Different letters denote statistically significant differences among transects (Mann-Whitney U test, p < 0.05). M: moderately impacted; S: strongly impacted.

		Olabeaga	**Rontegi**	**Lamiako**	**Inner Abra**
Sediment characterisation	Gravel	1.6 ± 2.1	0.0 ± 0.1	6.3 ± 8.6	0.1 ± 0.1
	Sand	83.9 ± 22.0	23.5 ± 4.3	74.3 ± 32.7	32.7 ± 8.3
	Mud	14.6 ± 22.4	76.5 ± 4.3	46.3 ± 40.9	67.2 ± 8.3
Metals	Cd	0.7 ± 0.2	1.1 ± 1.7	1.1 ± 1.8	1.0 ± 0.4
	Cr	70.9 ± 31.6	84.7 ± 28.1	101.4 ± 41.6	57.7 ± 8.2
	Cu	146.7 ± 48.7	68.2 ± 47.9	134.5 ± 573.1	69.2 ± 68.2
	Ni	61.9 ± 16.8	41.6 ± 11.2	48.2 ± 23.8	37.6 ± 2.8
	Hg	0.3 ± 0.6[a]	1.4 ± 0.8[a]	2.6 ± 8.0[b]	0.8 ± 0.2[a]
	Pb	127.5 ± 67.8	123.4 ± 94.8	230.4 ± 646.2	104.6 ± 18.0
	Zn	241.0 ± 149.3	345.3 ± 150.0	399.3 ± 661.2	274.7 ± 38.9
Organic compounds	ΣPCBs	35.1 ± 17.0[a]	256.4 ± 75.4[b]	278.0 ± 365.1[b]	252 ± 479.2[b]
	ΣPAHs	402 ± 158[a]	5851 ± 1909[b]	56040 ± 44560[b]	18720 ± 44316[b]
	ΣDDTs	3.0 ± 0.7	6.0 ± 23.8	5.0 ± 0.4	4.6 ± 0.4
SQG-Qs	Metals	0.9 (M)	1.0 (M)	2.9 (S)	0.8 (M)
	PCBs	1.1 (S)	8.1 (S)	13.7 (S)	13.3 (S)
	PAHs	0.3 (M)	3.5 (S)	36.3 (S)	21.4 (S)
	DDTs	0.1 (M)	0.3 (M)	0.2 (M)	0.3 (M)
	Metals + organic compounds	0.7 (M)	2.5 (S)	9.8 (S)	6.2 (S)

Note: HCHs were under detection limit; ΣPCBs: is the sum of 28, 52, 101, 118, 138, 153 and 180 congeners; ΣPAHs: is the sum of fluorene, naphthalene, anthracene, dibenz(a,h)anthracene, acenaphthene, acenaphthylene, phenantrene, pyrene, chrysene, benzo(e)pyrene, benzo(g,h,i)perylene, fluoranthene, benzo(a)anthracene, benzo(b)fluoranthene, benzo(a)pyrene, indeno(1,2,3-cd)pyrene; ∑DDTs is the sum of dichlorodiphenyldichloroethylene, dichlorodiphenyldichloroethane, dichloro diphenyltrichloroethane.

Additionally, sediments from all transects were moderately impacted by DDTs and strongly impacted by PCBs. On the other hand, the inner most area (Olabeaga) presented sediments moderately impacted by PAHs, while the rest of the sampling areas were strongly impacted. Overall, SQG-Qs determined in sediments collected along the Ibaizabal estuary during 2009 – 2013 indicated that the inner most part (Olabeaga) was classified as moderately impacted, while the rest of the estuary was classified as strongly impacted indicating that adverse biological effects are likely expected. Metals, PCBs and PAHs were the contaminants most likely causing adverse effects since their toxicological significance values were higher than 1.

3.2. Biometric parameters

The length of gobies of the Ibaizabal estuary ranged between 4.0 and 7.2 cm and the weight from 0.7 to 3.8 g, while the condition factor varied between 0.6 and 1.4 g cm^{-3}. Significant differences in length and weight were observed among campaigns and transects (Table 3.3), with gobies from Olabeaga and Inner Abra attaining larger sizes than those from Rontegi and Lamiako. However, no significant differences were obtained in K values (Table 3.3). The length of gobies did not show significant differences among campaigns. In contrast, the weight presented significantly higher values in 2011 (1.25g as mean value) than in 2012 (1.09g as mean value).

3.3. Metal bioaccumulation levels

Metal bioaccumulation levels measured in a pool of 30 individuals (whole body burden) per transect are shown in Table 3.4. In general, there were not remarkable differences in metal levels along the estuary and there was not a clear bioaccumulation gradient from upstream to downstream waters. However, except for the case of Pb at the Inner Abra, most of the metals presented lower bioaccumulation levels in 2013 than in 2012.

Table 3.3. Summary of the results from the non-parametric Bennett's bivariate sign test performed to analyse the effects of the campaign, transect and the interaction between these two factors on histopathological indices of different organs of gobies. DF: degrees of freedom; χ^2: Chi-square; *I*: histopathological index. Statistically significant differences are indicated.

Biological variables	**Factor**	**DF**	χ^2	***p***
Length	Campaign	1	0.0055	
	Transect	2	0.2244	< 0.05
	Campaign x transect	5	0.2501	
Weight	Campaign	2	0.0342	< 0.05
	Transect	1	0.2057	< 0.05
	Campaign x transect	5	0.0294	
K	Campaign	1	0.0011	
	Transect	2	0.0003	
	Campaign x transect	5	0.0014	
I_{Liver}	Campaign	1	0.0126	< 0.05
	Transect	2	0.1147	< 0.05
	Campaign x transect	5	0.0021	
I_{Gills}	Campaign	1	0.0063	< 0.05
	Transect	2	0.0002	
	Campaign x transect	5	0.0030	
I_{Kidney}	Campaign	1	0.0154	< 0.05
	Transect	2	0.0040	
	Campaign x transect	5	0.0265	
I_{Spleen}	Campaign	1	0.0018	
	Transect	2	0.1358	< 0.05
	Campaign x transect	5	0.0053	

Table 3.4. Metal bioaccumulation (mg kg^{-1} in ww) in the whole body of gobies collected from 2011 to 2013 along the Ibaizabal estuary. n.d.: no data

Year	**Transects**	**Cd**	**Cu**	**Pb**	**Hg**	**Zn**
2011	Olabeaga	0.01	1.06	0.05	0.08	25.43
	Rontegi	0.00	0.94	0.03	0.04	23.66
	Lamiako	n.d.	n.d.	n.d.	n.d.	n.d.
	Inner Abra	n.d.	n.d.	n.d.	n.d.	n.d.
2012	Olabeaga	0.17	2.16	0.03	0.01	29.54
	Rontegi	0.14	1.91	0.03	0.01	2.85
	Lamiako	0.23	2.71	0.05	0.02	29.70
	Inner Abra	0.17	2.35	0.03	0.01	12.72
2013	Olabeaga	0.01	0.89	0.05	0.01	11.53
	Rontegi	0.00	0.25	0.02	0.01	3.96
	Lamiako	0.01	0.75	0.07	0.01	12.25
	Inner Abra	0.00	0.34	0.17	0.01	2.61

3.4. Multi-organ description and prevalence of lesions

The normal hepatic structure of gobies was characterised by hepatocytes disposed in a simple layer aligned with sinusoids (Figure 3.2A). In several cases, pancreatic tissue was also shown throughout the hepatic parenchyma, especially surrounding blood vessels. Hepatic tissue of gobies collected along the Ibaizabal estuary was highly lipidic, presenting fairly large fatty vacuoles regularly distributed. Thus, nuclei of hepatocytes were commonly displaced to the periphery of the cells, becoming difficult to distinguish the normal hepatic lobular architecture. Therefore, progressive changes were the most prevalent reaction pattern along the Ibaizabal estuary; within which fat vacuolation of hepatocytes was the most frequent alteration (Figure 3.2B) showing prevalences up to 90%. Spongiosis hepatis (0 – 15%) and concentric periductal fibrosis of bile ducts (0 – 8%) were rarely recorded and all the cases were registered in gobies from the Inner Abra located in the outer most transect. Circulatory disturbances, such as haemorrhage (0 – 32%) (Figure 3.2B), hyperaemia (15 – 86%) and aneurysm (0 – 36%), presented rather predominant frequencies comprising the second most regular reaction pattern. Following closely, inflammatory responses, which include melanomacrophage centers (42 – 79%) and lymphocytic infiltration (0 – 21%), were recorded mainly in relation to circulatory disturbances or parasite infections. Within regressive changes, necrosis (0 – 5%) and hepatocellular nuclear pleomorphism (0 – 69%) were observed. A few parasites (< 5%), mainly nematodes, were recorded throughout the hepatic parenchyma.

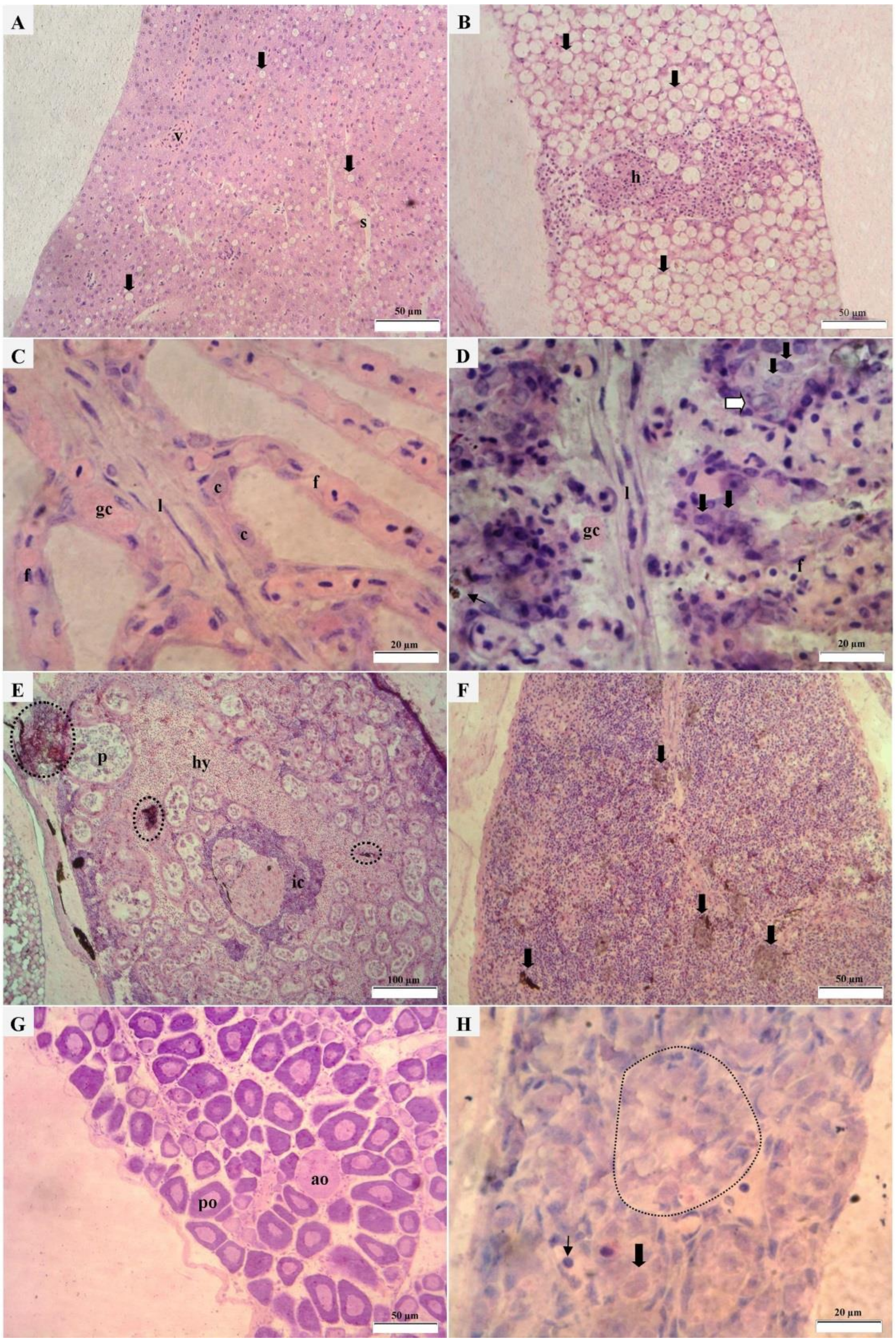

Figure 3.2. Different organs of gobies stained with haematoxylin and eosin (*A*) Normal hepatic tissue with marked sinusoids (s) and slight fat vacuolation of hepatocytes (wide arrows) in a goby from a minor disturbed estuary close to the Ibaizabal estuary. (*B*) Hepatic tissue affected by fat vacuolation (wide arrows) and haemorraghe in a goby from the Ibaizabal estuary (h). (*C*) Normal gills with well differentiated lamellae (l) and filaments (f) with chloride (c) and goblet (gc) cells. (*D*) Gills affected by hyperplasia (black arrows) and hypertrophy (white arrow) of chloride cells and degeneration of goblet cells (gc). (*E*) Renal tubules presenting myxospora-like parasites (p) surrounded by hyperaemia (hy) and melanomacrophage centers (circle). (*F*) Splenic tissue with high densities of melanomacrophage centers (wide arrows). (*G*) Female gonad tissue with oocytes in primary growth (po) showing an atretic oocyte (ao). (*H*) Male gonad with seminiferous lobule (dotted circle) in early gametogenesis showing spermatogonias (wide arrow) and spermatids (narrow arrow). v: blood vessel; l:branchial lamellae; f: branchial filaments.

The normal architecture of gills consists of lamellae attached to filaments. The single-cell thick lamellar epithelia contained goblet and chloride cells (Figure 3.2C). As in liver, progressive changes were the most prevalent reaction pattern in this organ; within which lamellar fusion (2 – 58%) was the most frequent alteration, although other lesions such as hyperplasia (0 – 37%) and hypertrophy (0 – 17%) of chloride cells, hyperplasia (0 – 46%), hypertrophy (0 – 50%) and degeneration (0 – 23%) of goblet cells were also observed (Figure 3.2D). Regressive changes were recorded at a lesser frequency than progressive changes and they were mainly located in filaments. The most predominant alteration was epithelial desquamation (28 – 67%), while epithelial lifting (0 – 53%) and necrosis (0 – 5%) were recorded at lower prevalence. Circulatory disturbances and inflammatory responses were related, and, occasionally, inflammatory alterations were associated with infection. The highest prevalence were retrieved for hyperaemia (0 – 85%) and MMCs (17 – 89%), although other alterations such as haemorrhage (0 – 30%) and lymphocytic infiltration (0 – 20%) were also observed at a lower frequency. The frequency of parasites (mainly Chlamydia-like and ciliates) in gills ranged between 0 – 26% of surveyed fish.

The kidney parenchyma was composed of renal tubules surrounded by hematopoietic interstitial tissue containing mainly erythrocytes and blast cells. In general, no predominant reaction pattern was observed neither along the estuary nor among sampling campaigns. MMCs (28 – 75%) showed one of the highest prevalence (Figure 3.2E), which was closely related with circulatory disturbances such as haemorrhage and hyperaemia (both 0 – 67%) (Figure 3.2E). Renal tubule regression characterised by the narrowing of the tubule lumen and the subsequent loss of tubule shape and morphology, showed prevalence values ranging from 14 to 75%. As a further and consequent alteration of renal tubule regression, necrosis was also observed at low prevalence (0 – 8%), showing loss of tubule organisation. Regarding progressive changes, hypertrophy, hyperplasia and hydropic vacuolation of epithelial cells of renal tubules were recorded with similar prevalence (0 – 34%). Infections were also observed with high frequencies; mostly myxospora-like parasites (50 – 100%) in the renal tubule lumen (Figure 3.2E), although infrequent cases of nematode presence also were recorded.

The normal splenic white pulp was composed of lymphoid tissue, while the red pulp held mostly fibroblasts supporting sinusoids, macrophages and lymphocytes. Gobies along the Ibaizabal estuary showed no obvious dominance of either splenic tissue. However, alterations in the frequency of MMCs were the most prevalent alteration (85 – 100%) along the estuary (Figure 3.2F). Haemorrhage was also observed at a lower frequency than MMCs (ranging between 15 – 80% of the fish) and no regression changes were present. In this lymphoid organ no infections were recorded.

The gonads of gobies of the Ibaizabal estuary presented early gametogenic stages in both male and females (Figures 2G and 2H). Oocyte atresia (0 – 39%) was punctually recorded. No evident intersex cases were found in male gobies collected along the Ibaizabal estuary. No parasitic infections were observed in the reproductive organs of either gender.

3.5. Multi-organ histopathological indices

According to the bivariate sign test, there were significant differences in multi-organ histopathological indices among transects and campaigns, while their interaction was not significant (Table 3.3). In this respect, the hepatic histopathological indices of gobies collected in 2013 along the estuary were significantly lower than those recorded in previous campaigns. On the other hand, significantly lower hepatic histopathological indices were recorded in gobies collected at Inner Abra in 2011 and 2013 than in the rest of the estuary in the same campaigns (Figure 3.3A). Similarly, the histopathological damage registered in gills and kidney of gobies collected in 2013 at Inner Abra was significantly lower than in previous campaigns. In the case of spleen, there were no histopathological differences among campaigns (Table 3.3), although there were significantly lower histopathological indices in gobies collected at Rontegi and Inner Abra in 2013 (Figure 3.3D). According to the estimated histopathological indices, target organs, from the most to the least affected, were ordered as follows: liver, gills, kidney and spleen (Figure 3.3).

Taking into account the mean values obtained in each campaign and transect, the histopathological indices of liver and gills (R = 0.7; $p < 0.05$, n = 11) and gills and kidney (R = 0.7; $p < 0.05$, n = 11) were significantly correlated. Additionally, there were significant correlations between Cd bioaccumulation levels and histopathological indices in liver (R = 0.7; $p < 0.05$, n = 10), gills (R = 0.7; $p < 0.05$, n = 10) and kidney (R = 0.7; $p < 0.05$, n = 10). Similarly, bioaccumulation of Cu was significantly correlated with histopathological indices in liver (R = 0.8; $p < 0.05$, n = 10) and gills (R = 0.9; $p < 0.05$, n = 10), while Zn bioaccumulation was correlated with liver histopathological indices (R = 0.7; $p < 0.05$, n = 10). Finally, histopathological indices were not significantly correlated with the SQG-Qs of sediments.

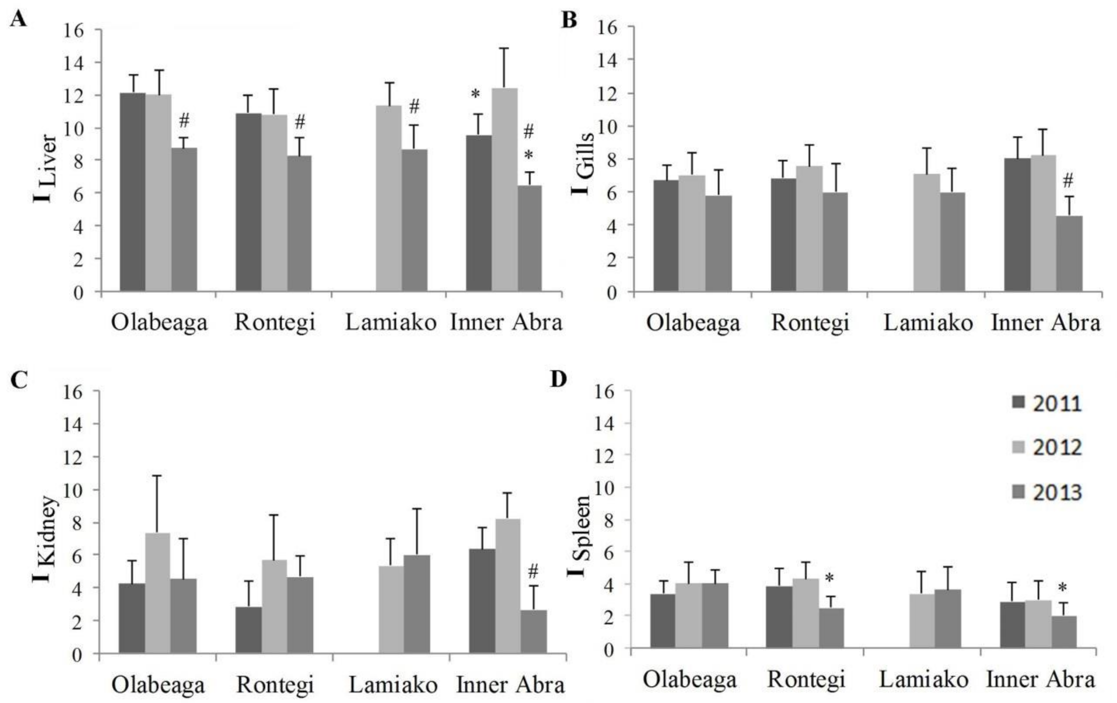

Figure 3.3. Histopathological indices of (*A*) the liver, (*B*) gills, (*C*) kidney and (*D*) spleen of gobies collected along the Ibaizabal estuary. (#) indicates statistically significant differences among campaigns in each transect and (*) among transects in each year (Mann-Whitney U test, $p < 0.05$). Error bars indicate 95% confidence intervals.

4. Discussion

The Ibaizabal estuary is generally regarded as a highly impacted by mixed organic and inorganic contaminants (Belzunce et al., 2004; Cearreta et al., 2000; Gredilla et al., 2013; Leorri et al., 2008). However, a general decrease of pollution levels and a subsequent improvement of the quality of water, sediments and biological communities have recently occurred (Borja et al., 2010; Díez et al., 2009; García-Barcina et al., 2006; Leorri et al., 2008; Pascual et al., 2012; Tueros et al., 2009). Nevertheless, the SQG-Qs revealed sediments strongly impacted by contaminants along the Ibaizabal estuary, which indicates that adverse biological effects are still expected. As exception, sediments from the inner most area (Olabeaga) were moderately impacted partly due to its lower industrial loads and its progressive recovery (Borja et al., 2006; Gredilla et al., 2013; Legorburu et al., 2014). More specifically, Lamiako showed sediments strongly impacted by metals, while Olabeaga, Rontegi and Inner Abra presented sediments moderately impacted. This gradient of sediment contamination by metals along the Ibaizabal estuary is consistent with that reported by Gredilla et al. (2013) in sediments collected from 2005 to 2010 within the same study area. Conversely, sediment contamination by metals was not in accordance with metal bioaccumulation levels determined in the whole tissue of gobies. Considering the short length (22 km) of the Ibaizabal estuary and the seasonal spawning migrations of gobies (see Bouchereau and Guelorget, 1998), metal bioaccumulation may not reflect site-specific sediment pollution levels, reflecting a global pollution status of the Ibaizabal estuary. On the other hand, metal bioaccumulation levels of gobies (*Pomatoschistus* spp.) from the Ibaizabal estuary were lower than the levels of *Pomatoschistus microps* and *Pomatoschistus minutus* collected from the Tagus estuary (Caçador et al., 2012). It must also be stressed that metal bioaccumulation was recorded as a general indicator of exposure. Nevertheless, further research is needed to disclose the ability to accumulate other toxicants such as organic compounds.

Previous studies highlighted the high level of pollution in the Ibaizabal estuary that may have an impact on resident aquatic organisms (Cearreta et al., 2000; Gredilla et al., 2013; Leorri et al., 2008). In agreement with the relatively similar toxicological significance of sediments and metal bioaccumulation of gobies, the multi-organ histopathological indices yielded a similar gradient along the estuary, which may reflect a global health status of the Ibaizabal estuary. Additionally, in accordance to the metal bioaccumulation, estimated multi-organ histopathological indices of gobies collected in 2013 presented generally lower histopathological damage levels. This lower affection seems to be more related with the amelioration of the area rather than with the differences in length between campaigns, since gobies of the same age cohort were used in the study.

The multi-organ histopathological indices of gobies revealed different affection degree in each target organ (liver > gills > kidney > spleen). This is probably owing to the specific function and sensitivity of each organ, which is highly related to its biological role, as noted by other authors (*e.g.* Costa et al., 2009, 2013). In this way, liver with a high detoxification role and gills with permanent contact with potential pollutants showed the most severe histopathological indices (Bernet et al., 1999). In agreement, gills of soles exposed to contaminated sediments were the most susceptible organ to acute effects exposure to waterbone pollutants, while liver was involved in chronic effects of accumulated contaminants (Costa et al., 2009). Kidney also participates in the metabolism of organic xenobiotic as part of ion excreting processes (Bernet et al., 1999; Costa et al., 2010); thus, this target organ presented higher histopathological indices than spleen, but lower than liver and gills. Lastly, spleen was the less sensitive organ to pollutants, showing fairly lower histopathological damage in fish collected along the estuary. Likewise, Costa et al. (2013) revealed a similar inter-organ affection pattern in soles after Cd exposure. Therefore, fish liver, gills and kidney are perhaps more suitable target organs for biomonitoring approaches, at least in similar circumstances (Adams and Sonne, 2013; Bernet et al., 1999; Costa et al., 2009). Spleen, however, may not be an appropriate target organ in gobies from the Ibaizabal estuary, although it has been previously shown that in other fish species this organ may be impaired as a consequence of xenobiotic exposure (Della Torre et al., 2010; Monteiro et al., 2006).

No pre- or neoplastic lesions were recorded in gobies collected in the Ibaizabal estuary, certainly owing to the relatively slow formation of neoplastic lesions (Myers et al., 1987) and the short life span of gobies (Dolbeth et al., 2007). Therefore, unlike for other benthic teleosts, especially flatfish, non-neoplastic alterations have been proposed as useful histopathological biomarkers for contaminant exposure in gobies (Stentiford et al., 2003). Fat vacuolation was the most prevalent alteration in liver of gobies involving a remarkable hepatic architectural degeneration. Still, it must be highlighted that the degree of relative condition of hepatocytes is highly dependent on the reproductive stage and the availability of an adequate food supply (Davies and Vethaak, 2012). However, gobies collected in minor disturbed estuaries close to the Ibaizabal estuary during the same period showed slight or absence of fat vacuoles (Revilla et al., 2014), indicating that gobies analysed in the present study have probably held this hepatocellular alteration as a consequence of exposure to pollutants. Fat vacuolation, which has been found related to metal and organic contaminants exposure (Greenfield et al., 2008; Triebskorn et al., 2008; van Dyk et al., 2007), was observed in almost all the specimens along the estuary, suggesting a similar impact within the estuary.

In gills, lamellar desquamation and fusion were the most recurrent lesions, which is consistent with other works that suggest gills as more susceptible organ to acute lesions caused by contaminants exposure (Costa et al., 2009; Oliveira Ribeiro et al., 2005). Progressive alterations in chloride and goblet cells were recorded as suitable traits for biomonitoring approaches in several species (Fanta et al., 2003; Monette et al., 2008) and these lesions have already been found to be linked to exposure to unspecific xenobiotics (Arellano et al., 1999; Costa et al., 2013; López-Galindo et al., 2010). In kidney, renal tubule alterations were the most frequent (hyperplasia and hypertrophy of epithelial cells, hydropic vacuolation and renal tubule regression). These renal alterations have also been described in studies focused on contaminants exposure (Beširović et al., 2010; Cengiz, 2006; Costa et al., 2010; Giari et al., 2007; Triebskorn et al., 2004). In spleen, MMCs were the most significant histopathological trait. Still, it has been suggested that this feature may not be fairly consistent for biomonitoring approaches (Costa et al., 2013). Since the major function of the spleen is the removal of degraded erythrocytes, the deposition of iron-containing proteins (hemosiderin and ferritin) is fairly common (Agius and Roberts 2003). Additionally, some authors have reported changes in MMC densities after contaminants exposure (Giari et al., 2007; Lemaire-Gony et al., 1995). Regarding gonads, gobies collected in autumn showed early gametogenic stages, which is in accordance with the reproductive cycle described in Iberian estuaries (Dolbeth et al., 2007). Besides, male gonads presented no intersex cases, from which low impact of xenoestrogenic compounds may be suspected, even though this subject needs further research for the area.

5. Conclusions

The present study showed that sediments from the Ibaizabal estuary ranged between strongly and moderately impacted by metals and organic compounds, indicating that adverse biological effects to the local biota could be expected. Metal bioaccumulation levels in whole organism of gobies revealed similar levels at all transects. However, metal bioaccumulation was seemingly reduced in more recent campaigns, which is consistent with the notion of a recovering estuary following recent attempts to ameliorate, *e.g.* wastewater treatment and management. Also, lower histopathological indices were recorded in liver, gills and kidney of fish collected during the most recent campaigns (held in 2013). Liver, gills and kidney attained higher histopathological indices than spleen, highlighting their implication in pollutant detoxification processes.

Fat vacuolation of hepatocytes, lamellar fusion and melanomacrophage centers were the most prevalent hepatic, branchial and renal alterations, respectively; indicating exposure to unspecified toxicants, although the influence of other unknown environmental facts should not be excluded. No severe pathological traits were registered in gonads, indicating undisturbed reproductive status. In conclusion, the use of multi-organ histopathology in gobies in combination with metal bioaccumulation and sediment contamination levels, contribute to a better understanding of sub lethal effects and a more accurate environmental risk assessment in the Ibaizabal estuary.

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Chapter 4

Histopathological indices in common sole and European hake for implementation of the European MSFD along the Basque Continental Shelf

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Summary

Common sole and European hake, together with sediments, were collected during two campaigns along the Basque continental shelf to study the utility of two existing histopathological indices for assessing the biological effects of contaminants to implement the European MSFD. Hepatic and gonad histopathology were measured, and metal and/or organic contaminants were determined in both liver and sediments. Sediments from the Basque continental shelf were found to be moderately impacted by metals but non-impacted by organic compounds. Metal bioaccumulation and histopathological lesions in the liver were higher in common sole than in European hake, although non-specific and early non-neoplastic toxicopathic lesions were observed in both species. No gross alterations were recorded in the gonad. The two histopathological indices applied were highly correlated in both organs, but the lack of correlation between sediment contamination levels, bioaccumulation and histopathological indices suggests that other factors, rather than pollution alone, are responsible for the biological effects observed.

1. Introduction

The European MSFD (Directive 2008/56/EC) aims to achieve or maintain GES in European marine waters by 2020. This directive contains the first conceptual and practical guidelines for the development of efficient monitoring programmes in order to determine the status of marine environments (Borja et al., 2010; Lyons et al., 2010). Thus, the MSFD bases GES on eleven qualitative descriptors, with Descriptor 8 being formulated as “Concentrations of contaminants are at levels not giving rise to pollution effects”, thereby implying that contamination is to be monitored and assessed in order to determine whether the levels of toxicants in the environment reach levels that are able to induce adverse effects in biota, *i.e*. becoming pollution. These contaminants that may cause harmful biological effects have been selected as priority chemical pollutants of EU-wide concern (Borja et al., 2010). The majority of these chemical substances are organic compounds (*e.g.* PCBs), although four metals (*i.e.* Cd, Ni, Pb and Hg) are also considered as priority substances (Directive 2008/105/EC, recently amended by Directive 2013/39/EU). The achievement of GES for Descriptor 8 relies on monitoring programmes covering the concentrations of chemical contaminants determined in different matrices, such as water, sediment or biota, and measuring the biological effects of pollutants on marine organisms (Law et al., 2010). According to the MSFD, such an assessment must be carried out for each marine region or subregion, for example the Bay of Biscay and Iberian coasts, within the North East Atlantic Ocean (Borja, 2006). Consequently, some authors have already proposed standardised sampling strategies using diverse sentinel organisms for biomonitoring in European marine waters within the scope of the MSFD (*e.g.* Benedetti et al., 2014; Costa et al., 2013). However, a detailed understanding of the responsiveness of potential target species towards toxicants and environmental parameters is required in order to achieve an adequate level of quality assurance (Lyons et al., 2010).

Flatfishes (Teleostei: Pleuronectiformes) have been widely employed as sentinel species, partly due to their benthic behaviour, which makes them appropriate organisms for biomonitoring marine sediments (Reynolds et al., 2003; Stentiford et al., 2003). Species such as dab (*Limanda limanda*), European flounder (*Platichthys flesus*) and English sole (*Pleuronectes vetulus*) are the main target species for monitoring purposes in Northern Europe and North-Western America (see for instance Köhler et al., 1992; Lang et al., 2006; Myers et al., 1998; Vethaak and Wester, 1996). However, as these species are less abundant around the Iberian Peninsula, other alternative species are likely to be more appropriate sentinels. One of the most abundant flatfish found on the Basque continental shelf (SE Bay of Biscay) is the common sole (*S. solea*), although the presence of Senegalese sole (*Solea senegalensis*) is also remarkable in offshore waters and estuaries (Franco et al., 2012; Quincoces et al., 2011). In this respect, adult common soles are characterised by a high habitat fidelity, thus making them a site-specific species (Pawson, 1995), although they do undergo short seasonal migrations from deeper offshore areas to shallower spawning grounds. The European hake (*M. merluccius*) has been also targeted as a sentinel organism for the Basque continental shelf, although to a lesser extent than flatfishes (Marigómez et al., 2006; Raingeard et al., 2009). The largest European hake nurseries are located in the Bay of Biscay (Casey and Pereiro, 1995), thus making this species an ideal candidate for biomonitoring approaches along the Basque continental shelf. European hake performs nictemeral migrations due to food availability; thus, it is a demersal species by day and pelagic at night (Sánchez and Gil, 2000). In addition, adults concentrate in canyons and on the rocky bottoms of the shelf break areas (Sánchez, 1993). As such, commercially exploited species, such as common sole and European hake, are of particular value for determining a plausible link between ecological and human risk.

Fish diseases and histopathological alterations are increasingly used as indicators of pollution effects since they provide a relevant biological end-point of historical exposure (Stentiford et al., 2003). In this regard, essentially qualitative histopathological approaches have provided vital information regarding the description of histological alterations in field-collected or tested aquatic organisms (*e.g.* Costa et al., 2009; Stehr et al., 1998). Nonetheless, the absence of some sort of quantification makes it difficult to establish statistically significant cause-effect relationships between pathology and contaminants and to assess the significance of the differences between areas or campaigns (Costa et al., 2009; Lang et al., 2006). Therefore, semi-quantitative histopathological approaches, especially those that consider the biological significance in addition to the dissemination of histopathological changes, may effectively circumvent this issue while conferring a wider degree of biological significance (Costa et al., 2009; Vethaak and Wester, 1996). Although different semi-quantitative histopathological indices are employed for the assessment of health status in target species (Bernet et al., 1999; Triebskorn et al., 2008; van Dyk et al., 2007), the present research is focused on the adaptation, application and comparison of two existing indices, one developed by Costa et al.(2009) for sole and another one by Lang et al. (2006) for flounder. Fish livers and gonads are considered to be ideal target organs for the assessment of biological effects caused by pollution due to their role in xenobiotic transformation, storage and elimination (Au, 2004; Bernet et al., 1999) and their relationship with the reproductive cycle and liability for upcoming generations (Blazer, 2002), respectively. Moreover, several emerging pollutants, known as endocrine disrupting chemicals, may affect the reproductive potential of gonads, thereby provoking alterations, such as intersex, that can be detected histologically (Bateman et al., 2004). In this work, the concentrations of some priority substances (according to the Directive 2008/105/EC, recently amended by Directive 2013/39/EU) with high potential to cause adverse biological effects have been assessed; metals have been measured both in the sediments and the fish tissues; and PCBs, PAHs and some organochloride pesticides were analysed in the sediments.

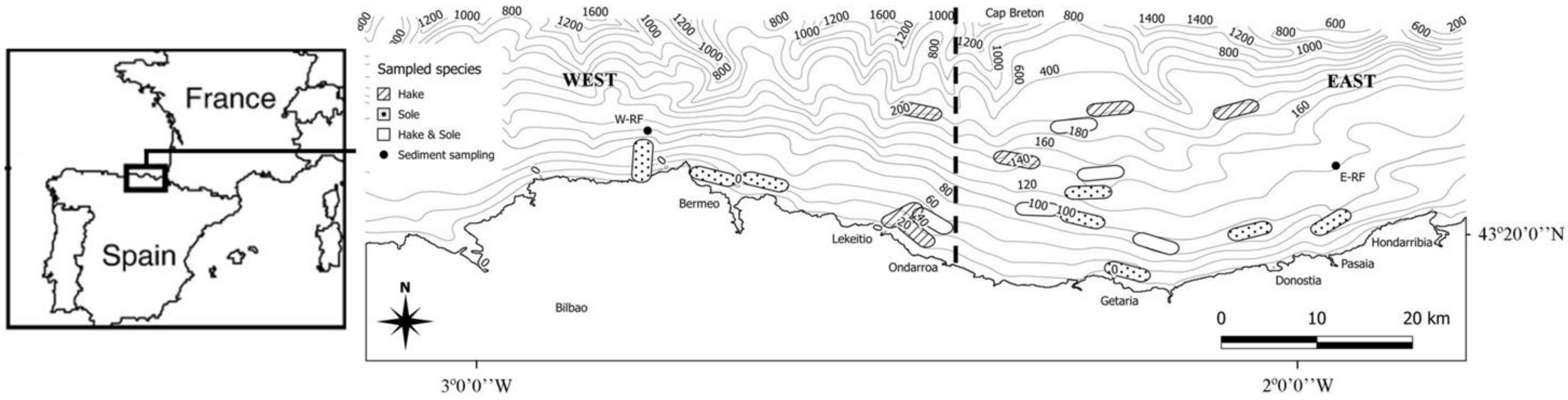
The present work aims to: (*1*) test potential sentinel fishes (common sole and European hake) for biomonitoring approaches along the North Atlantic Iberian shelf, namely in the Basque continental shelf; (*2*) adapt, apply and compare two existing histopathological indices: that developed by (Costa et al., 2009; Lang et al., 2006) in the liver and gonads of common sole and European hake; and (*3*) evaluate fish histopathology as an indicator of environmental pollution along the Basque continental shelf with regard to implementation of the European MSFD.

2. Material and methods

2.1. Sampling area and sample collection

The Basque continental shelf is located in the SE Bay of Biscay and its total length is approximately 150 km (Figure 4.1), with a width, which is characterised by its narrowness, in the range 7 – 20 km (Uriarte et al., 1998). Coastal waters in this region are influenced by both terrestrial climate and open ocean water masses (Valencia et al., 2004). Within the Basque continental shelf, soft bottom substrates below 100 m depth are dominated by mud and sand and occupy an area of 419 km^2 (Galparsoro et al., 2010). Human pressures (such as treated waste effluents) within the studied area are located inland, especially in the vicinity of estuaries (Borja et al., 2006). Nevertheless,

Figure 4.1. Location of sediment sampling sites (W-RF: West; E-RF: East) and fish collection areas (dotted: common sole; white: European hake and common sole; striped: European hake) along the Basque continental shelf.



there are additional pressures for offshore waters, such as a gas storage platform, four dredged sediment disposal sites and fisheries (Borja et al., 2011).

Sediment was collected annually during five years (from 2009 to 2013) with the aid of a Smith-McIntyre grab at two sampling sites, termed West and East Basque continental shelf (Figure 4.1). Common sole and European hake were collected during two years by bottom trawling in July 2012 and July 2013. A total of 30 individuals from each species were collected at each sampling site per campaign. Owing to the high mobility of European hake and the reduced width of the studied area, the Basque continental shelf was considered as a single overall sampling area. In the case of common sole, as a more restricted mobility was expected (Pawson, 1995), two sampling areas located in accordance with sediments in the West and East of the Basque continental shelf were considered independently. Fish were transferred to aerated water tanks where they were anesthetized and then their biometric parameters (length and weight), together with gross pathology through externally visible diseases, were registered on-board. Liver and gonad samples were subsequently excised. Two liver pieces were collected; one piece was frozen for subsequent bioaccumulation analyses, while the other one was processed immediately for histological assessment. Gonads were collected and fixed for histology. *Sagittae* otoliths were only removed from common sole for age determination. In the case of European hake, as there is no agreed protocol for otolith reading, the age was estimated according to the well-established length-age regressions (Piñeiro and Saínza, 2003).

2.2. Sediment characterisation and contamination levels

In order to determine the sediment granulometry, dried sediments were run through a column of sieves (gravel > 2 mm; sand 2mm – 63 lm; mud < 63 lm). Those sediment samples with low (< 10%) fine content were analysed by dry sieving (Folk, 1974), whereas sediment samples with higher fine fraction were determined using a LPDSA. Mud percentages calculated by LPDSA were transformed according to Rodriguez and Uriarte (2009) due to underestimation of the finest fraction of the sediment. Metal contents (mg kg^{-1} dry weight) were estimated in triplicate using the fine fraction of the sediment. Thus, dried sediment (1 g) was initially digested in an acid mixture (HCl:HNO3; 2:1) using a microwave system (MARS 5 Xpress CEM Corporation Instrument). The solid phases were then separated from the extracts by centrifugation and rinsed with deionised water. The metal concentrations in the extract (together with rinses) were determined by AAS (AAS800 Perkin Elmer): Cd was analysed using a THGA graphite furnace, with Zeeman background correction; Cr, Cu, Ni, Pb and Zn were determined in an air-acetylene flame; and Hg was measured using a quartz furnace AAS following the cold vapour method. Analytical accuracy was tested using a certified reference material (PACS-2, NRC). Recoveries for the certified metals were: 92% Cd; 70% Cr; 95% Cu; 95% Hg; 98% Ni; 100% Pb and 95% Zn.

Organic compounds such as PCBs (congeners: 28, 52, 101, 105, 118, 138, 153, 156 and 180), other organochloride pesticides (aldrin, dieldrin, isodrin, dichlorodiphenyldichloroethylene, dichlorodiphenyldichloroethane, dichloro diphenyltrichloroethane, hexachlorobenzene, b-hexachlorocyclohexane and c-hexachlorocyclohexane) and PAHs (fluorene, naphthalene, anthracene, dibenz(a,h)anthracene, acenaphthene, acenaphthylene, phenanthrene, pyrene, chrysene, benzo(e)pyrene, benzo(g,h,i)perylene, fluoranthene, benzo(a)anthracene, benzo(b)fluoranthene, benzo(a)pyrene, indeno(1,2,3-cd)pyrene) were also determined for the sediment as a whole. Thus, sediment samples (5–10 g) were extracted with a mixture of solvents (pentane:dicloromethane; 50:50) in an ASE (ASE 200, DIONEX).

The organic fraction was then purified by GPC and the different extracts collected, evaporated to dryness and reconstituted in isooctane for PCBs or in ethyl acetate for the remaining organochlorides. An 8 ml aliquot of sulfuric acid was added to the PAH extract prior to centrifugation. The organic phases were collected and analysed by GC-MS (Agilent 6890 GC coupled with an Agilent 5973 MSD instrument). Mean recoveries for almost all the certified PAHs were between 80 and 90%. Indeno[1,2,3-cd]pyrene and benzo[ghi]perylene showed lower recovery rates of between 60 and 70%. Mean recoveries, for almost all the certified PCBs, were between 85 – 110%, while for all the certified DDTs were between 60 – 90%.

To assess the toxicological significance of sediments, contaminant levels were compared to the TEL and PEL quality guidelines for sediments proposed by MacDonald et al. (1996). The risk of causing adverse biological effects was determined for each sediment according to the SQG-Qs described by Long and MacDonald (1998). Additionally, sediments were scored according to the risk of causing adverse biological effects as proposed by MacDonald et al. (2004): not impacted (<0.1); moderately impacted (0.1 – 1) and strongly impacted (>1).

2.3. Metal bioaccumulation levels

Metals were measured using a pool of 30 different liver pieces of hake or sole per sampling site and campaign for metal bioaccumulation analysis. Liver samples were digested in 10 ml of concentrated HNO3 in a microwave oven (MARS 5 Xpress CEM Corporation Instrument). After sample digestion, the metal content (mg kg^{-1} wet weight) was analysed by AAS (AAS800 Perkin Elmer). Hg was determined using the cold vapour technique and the remaining metals (Cd, Cu, Pb and Zn) using a graphite furnace equipped with a Zeeman background correction device. The analytical procedure was validated using shark muscle as certified reference material (DORM-2 from NRC). The mean recoveries were in the range of 80 – 90% for all of the metals, throughout the study.

2.4. Biometric parameters and externally visible diseases

Fish were measured (cm) and weighed (g) on-board and the condition factor (K) was subsequently calculated individually as [(total weight) × 100/(total length)^3] (g cm^{-3}). Externally visible diseases were determined according to ICES (1996).

2.5. Histology and histopathological procedure

Liver samples dissected out from the central axis were fixed in Davidson's fluid (10% formalin, 10% acetic acid and 30% ethanol) for 48 h, at room temperature. Gonads were fixed in 10% neutral phosphate-buffered formalin for 18 – 24 h at 4ºC. Samples were embedded in glycol methacrylate resin (Technovit 7100, Heraeus Kulzer) and nearly six sections with a thickness of 3 to 5 µm were obtained using a motorised rotary microtome (HM 350 S, Microm) equipped with a carbide tungsten knife. Sections were subsequently stained with H&E and at least three of them were examined under a light microscope (BX60, Olympus). The accuracy of histopathological assessment was checked by an operator from a series of blind reviews on the randomly-selected 30% of the pre-analysed samples and scoring did not diverge over 15% between pre-analysis and reviews.

The frequency or prevalence of histopathological alterations was estimated as (number of cases / total cases analysed) × 100 per sampling area in the case of liver and per sampling area and gender for gonad. Additionally, gonad development in sole was determined according to García-López et al. (2006) and in hake as proposed by Murua and Motos (2006).

The semi-quantitative histopathological approach in liver and gonad was performed following two different methods.

The first approach was based on the weighted histopathological indices developed by Bernet et al. (1999) and modified by Costa et al. (2009). The estimation of histopathological indices considers the relative biological importance (weight), to reflect the severity of each alteration, and the degree of dissemination of each lesion within the studied organ (score). The histopathological alterations were divided into four reaction patterns: (1) circulatory disturbances; (2) inflammatory responses; (3) regressive changes (implying functional loss); and (4) progressive changes (involving altered function). The global histopathological indices (*I*) were calculated for each individual and organ as follows:

$$Ih = \sum_{1}^{j} w_j \, a_{jh}$$

where w_j is the weight of the *j*th histopathological trait and a_j the score for the *j*th alteration of the individual *h*. The weights ranged from 1 to 3 (from minor, as in a circulatory disturbance, to high severity, such as necrosis).

The second semi-quantitative approach was based on the hepatic index proposed by Lang et al. (2006). Accordingly, histopathological lesions were classified into one of five main categories (Feist et al., 2004): (1) non-specific lesions; (2) early toxicopathic non-specific lesions; (3) pre-neoplastic lesions; (4) benign neoplasms; and (5) malignant neoplasms. The lesions recorded were further categorised into mild, moderate and severe depending on dissemination. If more than one alteration was recorded, the highest of the single scores was used.

2.6. Statistical analyses

Statistical analyses were carried out using the Statgraphics Plus 5 software package. The normality and homoscedasticity of the data was checked using the Shapiro Wilks and Levene tests, respectively. Whenever the assumptions for parametric analyses were not met, the non-parametric Kruskal-Wallis ANOVA by ranks was used to analyse differences in biometric data for common sole and European hake. In order to compare histopathological alteration frequencies between years and sampling areas, the Chi-squared test or Fisher's 2×2 Exact Test was performed, depending on the number of samples in each analysis. The Mann-Whitney U test was carried out to compare histopathological indices between years, sampling areas and genders. The non-parametric Spearman's rank-order R was used to assess correlations between biometric and histopathological variables. Multivariate statistics were obtained by correlation-based principal component analysis (PCA), integrating individual biological data (histopathological responses and biometric parameters), bioaccumulation and SQG-Qs. The significance level was set at $p < 0.05$ for all analyses.

3. Results

3.1. Sediment characterisation and contamination levels

Sediment granulometric characterisation, contamination levels and toxicological risk are shown in Table 4.1. The sediments from the West sampling site were mainly sandy, while those from the East were muddy. However, except for the higher Zn levels found

Table 4.1. Median and standard deviations for the granulometry (%) and metal (mg kg^{-1} dw) and organic (μg kg^{-1} dw) contents together with SQG-Qs for sediments collected from 2009 to 2013 in the West and East of the Basque continental shelf. * indicates statistically significant differences between sampling sites (Mann-Whitney U test, $p < 0.05$). M: moderately impacted; N: non-impacted.

Sediments		2009 – 2013	
		West	East
Granulometry	Gravel	23.8 ± 35.2	13.2 ± 29.5
	Sand	72.8 ± 33.7	18.6 ± 10.6
	Mud	3.4 ± 5.9	68.2 ± 19.4
Metals	Cd	0.3 ± 0.1	0.2 ± 0.1
	Cr	25.1 ± 4.7	32.9 ± 5.6
	Cu	23.5 ± 6.7	18.6 ± 2.9
	Fe	29883.5 ± 3955.6	29347.0 ± 4683.2
	Hg	0.6 ± 0.2	0.5 ± 0.1
	Ni	26.7 ± 5.9	27.3 ± 2.0
	Pb	79.9 ± 4.7	70.7 ± 18.6
	Zn	189.7 ± 18.5*	142.1 ± 19.3*
Organic compounds	ΣPCBs	1.4 ± 3.0*	9.7 ± 7.76*
	ΣPAHs	659.0 ± 505.7*	2032.1 ± 1097.8*
SQG-Qs	Metals	0.46 (M)	0.45 (M)
	ΣPCBs	0.02 (N)	0.03 (N)
	ΣPAHs	0.04 (N)	0.09 (N)
	∑PCBs+ ∑PAHs	0.03 (N)	0.07 (N)
	Metals + (∑PCBs+ ∑PAHs)	0.36 (M)	0.36 (M)

Note: organochlorides were below detection limit; ΣPCBs is the sum of 28, 52, 101, 105, 118, 138, 153, 156 and 180 congeners; ΣPAHs is the sum of fluorene, naphthalene, anthracene, dibenz(a,h)anthracene, acenaphthene, acenaphthylene, phenanthrene, pyrene, chrysene, benzo(e)pyrene, benzo(g,h,i)perylene, fluoranthene, benzo(a)anthracene, benzo(b)fluoranthene, benzo(a)pyrene, indeno(1,2,3-cd)pyrene.

in the West, there were no significant differences in sediment metal levels between the two sites. As regards organic compounds, significantly higher ΣPCBs and ΣPAHs levels were found in the East than in the West. Organochloride pesticides were below the detection limit in the Basque continental shelf. Toxicologically, metals were below TEL in 54% of the samples, whilst only Ni and Pb were over PEL values in the West. For organic compounds, 93% of the cases were below TEL values and no cases exceeded PEL. Thus, according to the SQG-Q estimation, sediments were classified as moderately impacted by metals and non-impacted by organic compounds. The SQG-Q for total contaminant content was also estimated for both areas and showed that, in general, the Basque continental shelf can be considered to be a moderately impacted area.

3.2. Metal bioaccumulation levels

The metal bioaccumulation levels determined in the liver of common sole and European hake are shown in Table 4.2. The metal content in liver was higher in common sole than in European hake, especially for Cu, Pb and Hg, whereas Cd and Zn levels were similar in both species. Accordingly, similar metal levels were recorded in both species for both campaigns. In the case of common sole, in general, there were no spatial differences between the western and eastern area.

Table 4.2. Bioaccumulation levels of metals (mg kg^{-1} ww) in pooled liver samples of common sole and European hake from the Basque continental shelf.

Species	Sampling área	Year	Cd	Cu	Pb	Hg	Zn
Common sole	West	2012	0.12	118.89	0.32	0.34	57.04
		2013	0.14	127.11	0.24	0.41	28.67
	East	2012	0.07	96.82	0.24	0.90	29.92
		2013	0.06	79.61	0.16	0.79	23.00
European hake	Basque continental shelf	2012	0.03	2.03	0.03	0.04	32.10
		2013	0.09	3.70	0.04	0.07	28.21

3.3. Biometric parameters and externally visible diseases

The length of common sole ranged between 32 and 36 cm and the weight between 393 and 520 g. The K fluctuated between 0.9 and 1.0 g cm^{-3}. The age of common sole corresponded to 2 – 4 years (Table 4.3). Although the weight of common sole from the West was significantly higher in 2012 than in 2013, no significant differences were

Table 4.3. Average and standard deviations for biometric parameters and prevalence (%) of hepatic alterations for each reaction pattern in common sole and European hake collected in 2012 and 2013. Different Latin letters indicate statistically significant differences between years and different Greek letters indicate significant spatial differences between sole from the West and East of the Basque continental shelf (Chi-squared test or Fisher's 2 × 2 Exact test, $p < 0.05$).

	Common sole				European hake	
Year	2012		2013		2012	2013
Sampling area	West	East	West	East	Basque continental shelf	
n	27	22	30	30	30	30
Length (cm)	36.6 ± 0.9	35.0 ± 4.4	33.8 ± 5.4	33.2 ± 4.7	33.8 ± 3.3^{a}	43.40 ± 3.5^{b}
Weight (g)	519.8 ± 220.4^{a}	461.0 ± 151.0	402.1 ± 194.3^{b}	393.3 ± 181.8	301.7 ± 82.1^{a}	574.2 ± 177.2^{b}
Condition factor (K)	1.0 ± 0.4	1.0 ± 0.1	0.9 ± 0.2	1.0 ± 0.1	0.8 ± 0.07^{a}	0.69 ± 0.1^{b}
Age (years)	2.6 ± 0.9	3.0 ± 0.9	2.6 ± 1.3	2.77 ± 1.0	2-3	2-3
1. Circulatory disturbance						
Hyperaemia	37.0	59.1	20.0	16.7	20.0	16.7
Haemorrhage	0.0	0.0	0.0	6.7	6.7^{a}	30.0^{b}
2. Inflammatory response						
MMCs	55.6	50.0	56.7	56.7	73.3	86.7
Lymphocytic infiltration	33.3	27.3	53.3	43.3	0.0	10.0
3. Regressive changes						
Necrosis	0.0	0.0^{a}	13.3	20.0^{b}	0.0^{a}	16.7^{b}
HNP	0.0	0.0	6.7	3.3	0.0	3.3
4. Progressive changes						
Spongiosis hepatis	48.2^{a}	31.8^{a}	$20.0^{b,\alpha}$	$60.0^{b,\beta}$	0.0	0.0
Fat vacuolation of hepatocytes	14.8^{a}	9.1	$50.0^{b,\alpha}$	23.3^{β}	43.3	53.3
Concentric periductal fibrosis of bile duct	11.1	9.1	16.7	13.3	13.3^{a}	26.7^{b}
HV of bile duct epithelial cells	0.0	0.0	6.7	6.7	0.0	6.7

n: sample number, MMCs: Melanomacrophage Centres, HNP: Hepatocellular Nuclear Pleomorphism, HV: Hydropic vacuolation found in length and K between sampling areas and campaigns (Table 4.3). In the case of European hake, the length ranged between 34 and 43 cm and the weight between 302 and 574 g. The condition factor ranged between 0.7 and 0.8 g cm^{-3}. No significant differences were observed between genders in any biometric variables for European hake, although significantly higher values were recorded for all biometric parameters in 2013 than in 2012. According to the commonly employed length-age relationship for European hake, the age of the individuals collected ranged between 2 and 3 years.

The externally visible diseases recorded in common sole were chiefly related to skin pigmentation, specifically ventral hyperpigmentation and dorsal depigmentation. However, the frequency of these anomalies was relatively low. Hyperpigmentation was found in 20% and 27% of common sole from the West and in 9% and 27% of common sole from the East collected in 2012 and 2013, respectively. Depigmentation, with slight dissemination, was only observed in 10% of common sole from the West in 2012. No signs of gross external pathologies, such as infections, ulcers or tumours, were found in common sole. European hake showed no signs of significant externally visible diseases, with the exception of minor cases of broken fins and skin injuries that may have been caused by fishing gear. No macroscopic nodules or abnormalities were recorded in the liver of either species.

3.4. Histopathological analyses

3.4.1. Histological description of the liver and gonad in common sole

Hepatocytes showed regular sized nuclei located in the centre of the cytoplasm. Hepatocytes were distributed in a layer aligned with sinusoids, branching out of larger blood vessels (Figure 4.2A). A few erythrocytes were commonly observed in blood vessels and sinusoids. Pancreatic tissue was also discerned within hepatic parenchyma.

Inflammatory and progressive changes, followed by circulatory disturbances, were the most frequent reaction patterns in fish from both sampling areas (Table 4.3). Hyperaemia was the most prevalent circulatory disturbance in common sole and was characterised by the congestion of blood vessels, with evident swelling (Figure 4.2E). Haemorrhage, however, was restricted to small foci, typically around blood vessels. MMCs were frequently observed in the periportal region (Figure 4.2C). Occasionally, MMCs were also observed near degenerated areas rich in Kupfer cells. In agreement with this, lymphocytic infiltration was also noted in the surroundings of vessels although, in a few cases, lymphocytes and inflammatory cells such as macrophages were also seemingly associated with parasites (especially trematodes and nematodes). Necrosis was infrequent and mainly found in the vicinity of portal vessels. Nuclear pleomorphism was also infrequent. Fat vacuolation was characterised by regularly disseminated vacuoles (no marked architectural and morphological distortion) within the parenchyma (Figure 4.2B). Hydropic vacuolation of biliary epithelial cells was present in bile ducts highly affected by concentric periductal fibrosis (Figure 4.2C). The surrounding tissue of vessels was affected by spongiosis hepatis, although this pathology could attain a diffuse condition in severe stages (Figure 4.2D). There was no evidence of either benign or malignant neoplasms. Overall, no significant spatial differences were recorded in any of the alterations analysed. However, in common soles collected in 2013, spongiosis hepatis was more prevalent in those from the East, whereas fat vacuolation was more frequent in fish from the West (Table 4.3).

Both genders revealed early-mid gametogenic stages in both the 2012 and 2013 campaigns (Figures 4.2F and 4.2H). Females presented gonads mainly in primary growth (≈ 90% of individuals), although cortical alveoli and hydration stages were also observed (≈ 5%) (Figures 4.2F and 4.2G). In 2012, males were mainly in early

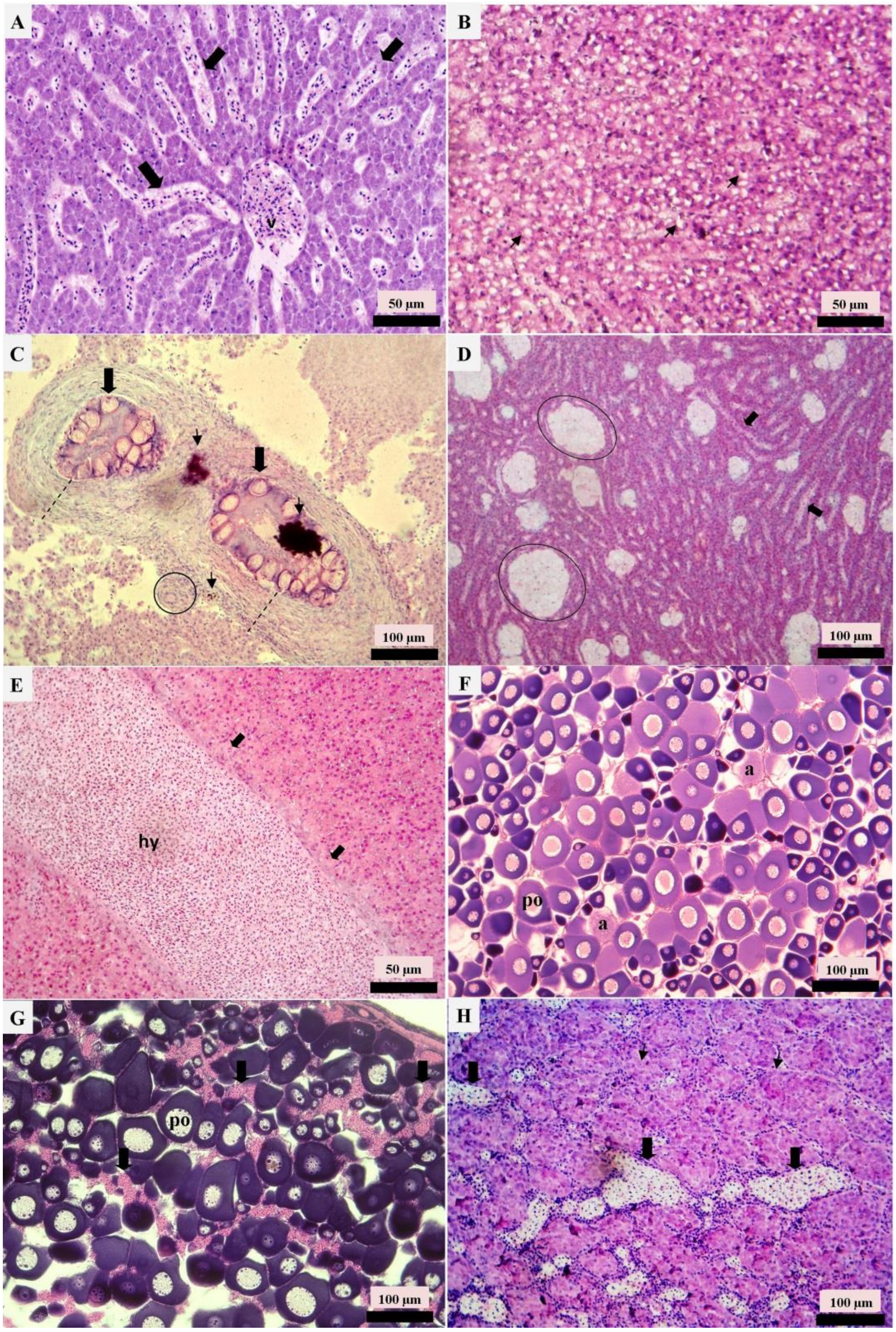


Figure 4.2. Hepatic and gonadal sections of common sole stained with haematoxylin and eosin. (*A*) Normal hepatic tissue with marked sinusoids (wide arrow) branching out to a blood vessel (v). (*B*) Hepatic fat vacuolation showing small vacuoles (narrow arrow). (*C*) Hepatic tissue presenting an advanced concentric periductal fibrosis (dotted line) and a hydropic vacuolation of bile duct epithelial cells (wide arrows). The normal bile duct is indicated by a circle, while MMCs are marked by narrow arrows. (*D*) Severe spongiosis hepatis marked by an ellipse and sinusoids by wide arrows. (*E*) Hepatic tissue showing hyperaemia (hy) within a blood vessel (wide arrows). (*F*) Normal primary growth oocytes (po) with the presence of atretic oocytes (a). (*G*) Female gonad tissue showing primary growth oocytes (po) affected by circulatory disturbance (wide arrows). (*H*) Male gonad tissue in early spermatogenesis showing a spermatogonia (narrow arrow) affected by circulatory disturbance (wide arrows).

gametogenesis (≈ 70%), whereas in 2013 most of the males were in mid gametogenesis (≈ 70%). Circulatory disturbances and inflammatory responses were the most remarkable reaction patterns in the gonads of both genders. Hyperaemia was the only circulatory disturbance recorded, usually within seminal lobes and often related to the infiltration of immune cells (Figures 4.2G and 4.2H). MMCs were frequently noted in connective tissue (such as in the medulla of testes) and, sporadically, in regions of deteriorated or necrotic tissue. Overall, MMCs were more frequent in males than in females (Table 4.4). Within regressive changes, a few cases of necrosis were observed in the gonads of both genders and, in females, oocyte atresia was occasionally present during early gametogenic stages. There was no evidence of intersex in males. Progressive changes were only recorded in females and appeared as lipid droplets in oocytes. Significant differences between sampling areas and campaigns were only observed in females. Thus, common soles from the West presented a higher prevalence of atresia in 2012 than in 2013, although hyperaemia was less frequent (Table 4.4).

3.4.2. Histological description of the liver and gonad in European hake

The normal hepatic structure of European hake was characterised by hepatocytes in a simple layer aligned with sinusoids (Figure 4.3A). As in common sole, in several cases pancreatic tissue was also observed throughout hepatic parenchyma. The hepatic tissue of European hake was highly lipidic, with vacuoles occupying almost all the cell volume and displacing the nucleus to the periphery (Figure 4.3A). Inflammatory and progressive alterations were the most prevalent reaction patterns (Table 4.2). Circulatory disturbances, such as hyperaemia and haemorrhagic foci, were also observed, with overall frequencies lower than 30% (Figure 4.3C, Table 4.2). MMCs generally appeared near the portal area (Figure 4.3D) and were occasionally related to

Table 4.4. Prevalence (%) of gonadal alterations for each reaction pattern by gender (female (F) and male (M)) in common sole and European hake collected in 2012 and 2013. Different Latin letters indicate statistically significant differences between years and different Greek letters indicate significant spatial differences between common sole from the West and East of the Basque continental shelf (Chi-squared test or Fisher's 2 × 2 Exact test, $p < 0.05$).

	Common sole								European hake			
Year	2012				2013				2012		2013	
Sampling area	West		East		West		East		Basque continental shelf			
Gender	F	M	F	M	F	M	F	M	F	M	F	M
n	16	10	10	11	16	14	21	9	13	13	18	12
1. Circulatory responses												
Hyperaemia	0.0 ^{a}	10.0	0.00	9.1	56.3 $^{b,\alpha}$	42.9	23.8 $^{\beta}$	11.1	15.4	0.0	33.3	25.0
2. Inflammatory responses												
MMCs	7.1	40.0	40.0	45.5	25.0	21.4	28.6	44.4	15.4	15.4	16.7	33.3
Lymphocytic infiltration	0.0	0.0	0.0	0.0	6.3	14.3	0.0	0.0	0.0	0.0	11.1	0.0
3. Regressive changes												
Necrosis	0.0	0.0	0.0	0.0	6.3	14.3	14.3	5.3	7.7	0.0	5.6	0.0
Pyknotic oocytes/spermatocytes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	22.2	0.0
Atresia/Intersex	35.7 ^{a}	0.0	30.0	0.0	6.3 ^{b}	0.0	19.1	0.0	15.4	0.0	16.7	0.0
4. Progressive changes												
Lipid in oocytes	0.0	0.0	0.0	0.0	6.3	0.0	28.6	0.0	0.0	0.0	0.0	0.0

n: sample number; MMCs: Melanomacrophage centres parasites (especially nematodes, 17 – 37%) (Figure 4.3D). Lymphocytic infiltration occurred mainly in the surrounding areas of vessels, similarly to MMCs, although they rarely appeared in hepatic parenchyma associated with parasites or highly deteriorated tissue. Among regressive alterations, necrosis and nuclear pleomorphism were rare (Table 4.2). Concentric periductal fibrosis (Figure 4.3B) and hydropic vacuolation of biliary epithelial cells (often associated) were observed in fairly low prevalence (Table 4.2). Haemorrhage, necrosis and concentric periductal fibrosis differed significantly between campaigns, presenting higher prevalence values in 2013 than in 2012 (Table 2).

Gonads showed early gametogenic stages, presenting oocytes in primary growth in all females (Figure 4.3E) and early-mid spermatogenic stage in around 80% of males (Figure 4.3G). Within circulatory disturbances, hyperaemia was more frequent in females than in males (Figures 4.3F and 4.3H). Only two inflammatory alterations, namely MMCs and lymphocytic infiltration, were recorded (Table 4.4). MMCs were more prevalent in males than in females, whereas lymphocytic infiltration was only noted in females collected in 2013. Regressive changes were only observed in female.gonads. Oocyte necrosis and pyknotic oocytes were observed rarely, and atresia was recorded in early stage oocytes, with similar prevalence values (15.4 – 16.7%) in both sampling campaigns (Table 4.4). No progressive changes were detected. Accordingly, no significant differences in gonad alterations were observed between campaigns and genders (Table 4.4).

3.5. Hepatic and gonad histopathological indices

The hepatic and gonad histopathological indices calculated for common sole and European hake, as proposed by Costa et al. (2009) and Lang et al. (2006), are illustrated in Figure 4.4.

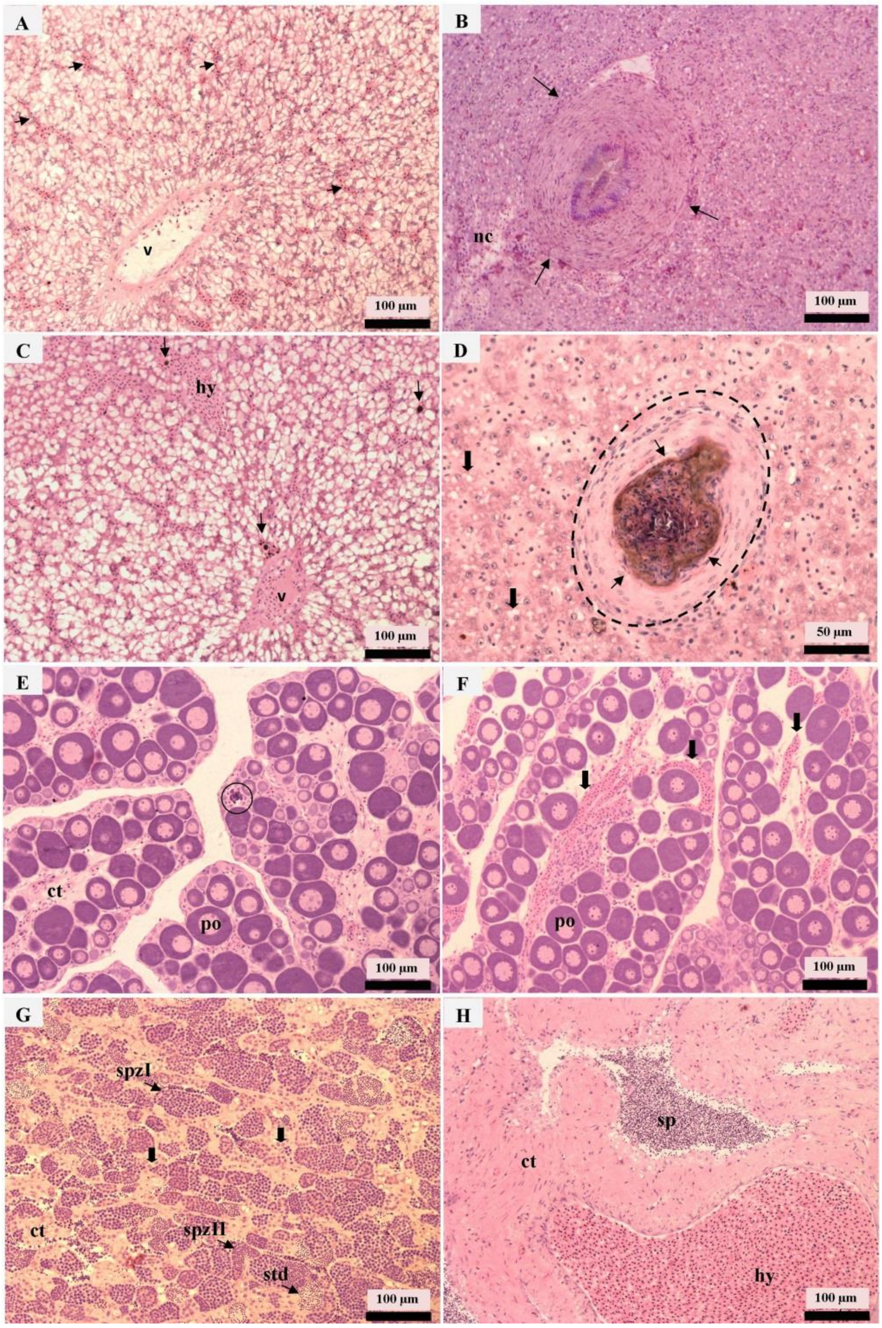


Figure 4.3. Hepatic and gonadal sections of European hake stained with haematoxylin and eosin. (*A*) Mild fat vacuolation showing hepatocytes disposed in sinusoids (arrows) branching out to a blood vessel (v). (*B*) Concentric periductal fibrosis in a bile duct (arrows). (*C*) Hyperaemia (hy) and MMCs (arrows) close to blood vessels (v) in hepatic parenchyma with a moderate fat vacuolation. (*D*) A parasite (circle) surrounded by compressed tissue and MMCs (arrows) in a hepatic parenchyma affected by mild fat vacuolation (white arrows). (*E*) An apoptotic oocyte (circle) in normal female gonad tissue with oocytes in the primary growth stage (po) surrounded by connective tissue (ct). (*F*) Female gonad tissue showing primary growth oocytes (po) affected by circulatory disturbance (narrow arrows). (*G*) Male gonad lobules in different gametogenesis stages (spermatogonia: wide arrows; spd: spermatida; spzI: primary spermatocytes; spzII: secondary spermatocytes). (*H*) Male gonad with spermatozoa (sp) in the medulla zone and a hyperaemia (hy) characterised by the occlusion of the blood vessel. ct: connective tissue; nc: necrosis.

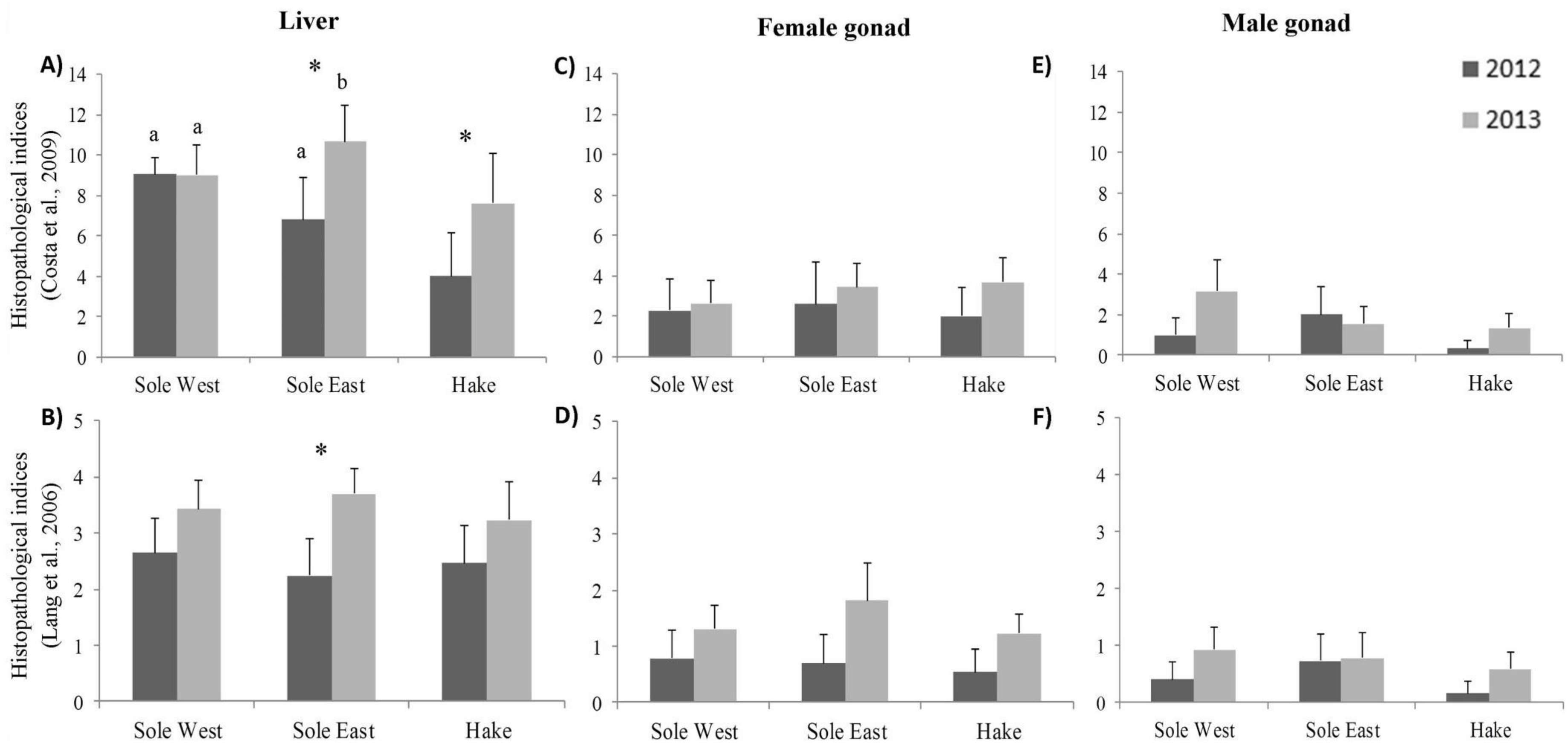


Figure 4.4. Histopathological indices in common sole and European hake collected in 2012 and 2013. Hepatic histopathological indices calculated according to (*A*) Costa et al. (2009) and (*B*) Lang et al. (2006); female gonadal histopathological indices calculated according to (*C*) Costa et al. (2009) and (*D*) Lang et al. (2006); male gonadal histopathological indices estimated according to (*E*) Costa et al. (2009) and (*F*) Lang et al. (2006). Error bars represent 95% confidence intervals. Different letters denote statistically significant differences among sampling areas for common sole and * indicates statistically significant differences between campaigns (Mann-Whitney U test, $p < 0.05$).

According to the index developed by Costa et al. (2009), hepatic histopathological indices were slightly higher in common sole than in European hake. No spatial differences were recorded in hepatic histopathological indices between common soles from the West and East in 2012, whereas in 2013 common soles from the East presented significantly higher values than common soles from the West (Figure 4.4A). Significant inter-campaign differences in hepatic histopathological indices were observed in European hake and common sole from the East, with the values for 2013 being higher than those for 2012. In contrast, no variations were recorded in either campaign in common soles from the West (Figure 4.4A). Gonad histopathological indices showed no significant differences between sampling areas in common sole or between campaigns in either species (Figures 4.4C and 4.4E). Similarly, no significant differences between genders were observed in either common sole or European hake.

According to the index developed by Lang et al. (2006), no significant differences were observed in hepatic histopathological indices between species (Figure 4.4B). However, although no spatial differences were recorded in hepatic histopathological indices for common sole, significantly higher values were registered in common sole from the East in 2013 than in 2012. In gonads, the histopathological indices determined in males and females tended to follow the same pattern observed when using the methodology developed by Costa et al. (2009), with no statistically significant differences between campaigns, areas or species (Figures 4.4D and 4.4F). Furthermore, both hepatic and gonad alterations were categorised as non-specific lesions in both species.

Table 4.5. Average (± 95% confidence intervals) histopathological condition indices for liver per reaction pattern for each area and year calculated according to Costa et al. (2009); and respective alteration weight (w) in common sole and European hake from the Basque continental shelf. Different Latin letters indicate significant differences between 2012 and 2013 (Mann-Whitney U test, $p < 0.05$).

LIVER			Common sole				European hake	
			2012		2013		2012	2013
Reaction pattern	Alteration	w	West	East	West	East	Basque continental shelf	
Circulatory disturbance	Hyperaemia Haemorrhage	1 1	1.0 ± 0.6	1.4 ± 0.6 [a]	0.5 ± 0.4	0.5 ± 0.4 [b]	0.5 ± 0.4	0.9 ± 0.5
Inflammatory response	MMCs Lymphocytic infiltration	1 2	3.4 ± 1.3	2.91 ± 1.1	4.0 ± 1.1	3.6 ± 1.3	1.7 ± 0.5	2.5 ± 0.6
Regressive changes	Necrosis HNP	3 2	0.0 [a]	0.0 [a]	1.1 ± 0.8 [b]	1.7 ± 1.3 [b]	0.0 [a]	1.1 ± 0.8 [b]
Progressive changes	Spongiosis hepatis Fat vacuolation of hepatocytes Concentric periductal fibrosis of bile duct HV of epithelial cells of bile ducts	1 2 2 2	4.6 ± 1.7	2.6 ± 1.4 [a]	3.5 ± 1.4	4.9 ± 1.5 [b]	1.7 ± 0.8	3.1 ± 1.2

Table 4.6. Average (± 95% confidence intervals) histopathological condition indices for gonad (female (F) and males (M)) per reaction pattern for each area and year calculated according to Costa et al. (2009), and respective alteration weight (w) in common sole and European hake from the Basque continental shelf. Different Latin letters indicate significant differences between 2012 and 2013 and different Greek letters indicate spatial differences in common sole (Mann-Whitney U test, $p < 0.05$).

GONAD			Common sole								European hake			
			2012				2013				2012		2013	
			West		East		West		East		Basque continental shelf			
Reaction pattern	Alteration	w	F	M	F	M	F	M	F	M	F	M	F	M
Circulatory disturbance	Hyperaemia	1	0.00^{a}	0.2±0.4	0.00	0.2±0.4	1.1±0.5^{b}	1.1±0.8	0.5±0.4	0.2±0.4	0.3±0.4	0.0	0.7±0.5	0.7±0.7
Inflammatory response	MMCs	1	0.1±0.3$^{a,\alpha}$	0.8±0.6	0.8±0.6$^{\beta}$	1.8±1.4	0.8±0.6^{b}	1.4±1.3	0.6±0.4	0.9±0.7	0.3±0.4	0.3±0.4	0.8±0.6	0.7±0.6
	Lymphocytic infiltration (only for common sole)	2												
Regressive changes	Necrosis	3	2.1±1.6	0.0	1.8±1.8	0.0	0.6±0.2	0.6±0.8	1.7±1.3	0.4±0.9	1.4±1.4	0.0	2.2±1.2	0.0
	Pyknotic oocytes/ spermatocytes	2												
	Atresia/Intersex	3												
Progressive changes	Lipid in oocytes (only for common sole)	1	0.0	0.0	0.0	0.0	0.1±0.0$^{\alpha}$	0.0	0.7±0.5$^{\beta}$	0.0	0.0	0.0	0.0	0.0

These two histopathological index approaches were significantly correlated in the liver of common sole (R = 0.71, $p < 0.05$, n = 97) and European hake (R = 0.67, $p < 0.05$, n = 60). In gonads, they were significantly correlated when combining both genders in common sole (R = 0.89, $p < 0.05$, n = 97) and European hake (R = 0.90, $p < 0.05$, n = 60). However, there was no significant correlation between liver and gonad for either of the histopathological indices.

With regard to the hepatic reaction patterns of the histopathological indices estimated according to Costa et al. (2009), common sole did not present significant differences between sampling areas (Table 4.5). Circulatory disturbances were significantly higher in common soles from the East in 2012 than in 2013, whereas regressive changes presented significantly higher indices in 2013 than in 2012 in both species (Table 4.5). Inflammatory changes in common sole female gonads showed significantly higher values in fishfrom the East than from the West in 2012, whereas progressive changes showed significantly higher values in common soles from the West than from the East in 2013 (Table 4.6). Only inflammatory and circulatory responses revealed significantly higher indices in 2013 than in 2012 (Table 4.6). In male common sole, there were no significant differences between either sampling areas or sampling campaigns (Table 4.6). Moreover, inflammatory responses (R = 0.61, $p < 0.05$, n = 97) and progressive changes (R = 0.77, $p < 0.05$, n = 97) were significantly correlated with hepatic histopathological indices in common sole (Table 5 and4. Figure 4.4), whereas regressive changes showed a high correlation with histopathological indices in gonad (R = 0.63, $p < 0.05$, n = 97) (Table 4.6 and Figure 4.4). In European hake, only progressive changes showed a significant correlation with hepatic histopathological indices (R = 0.77, $p < 0.05$, n = 60) (Table 4.5 and Figure 4.4).

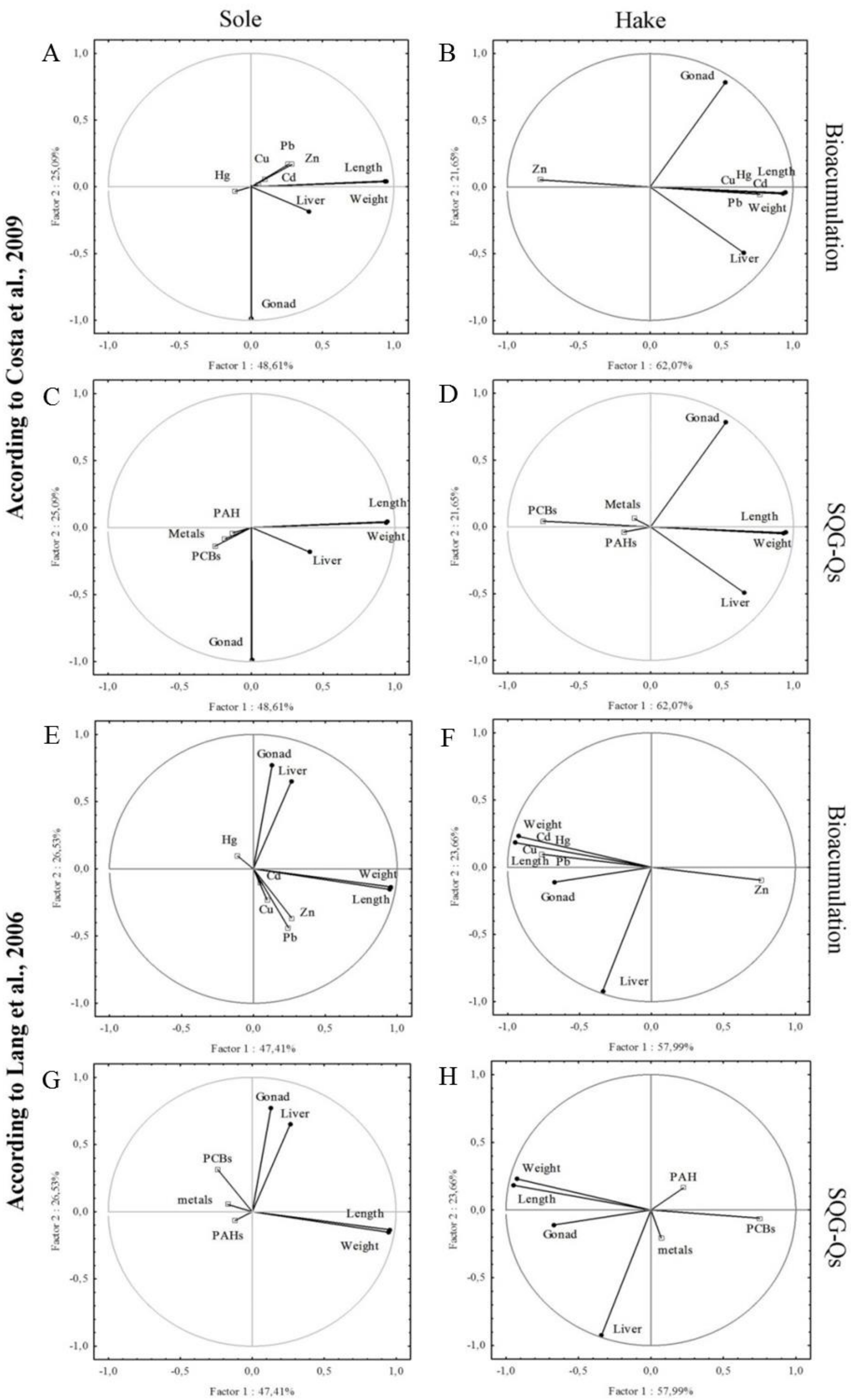


Figure 4.5. Principal component analysis for (*A*) common sole and (*B*) European hake integrating the global histopathological indices for the liver and gonad proposed by Costa et al. (2009), biometric parameters and hepatic metal-bioaccumulation levels. Principal component analysis for (*C*) common sole and (*D*) European hake integrating the global histopathological indices for the liver and gonad proposed by Costa et al. (2009), biometric parameters and SQG-Q for metals, PAHs and PCBs. The same principal component analyses are shown in *E-H* according to the histopathological indices proposed by Lang et al. (2006).

Correlation-based PCA, combining hepatic and gonad histopathological indices and biometric parameters for all individuals, together with metal bioaccumulation in liver (Figure 4.5A, 4.5B, 4.5E and 4.5F), gave two main factors that explained about 75% of the total variance for common sole and about 85% for European hake. Similar results were obtained using a second model, which combined hepatic and gonad histopathological indices estimated according to Costa et al. (2009) and Lang et al. (2006) and biometric parameters for all individuals together with the SQG-Qs (Figure 4.5C, 4.5D, 4.5G and 4.5H). Overall, the first PCA showed no correlation between metal bioaccumulation, biometric data and histopathological indices in common sole. Similarly, no clear correlation was evidenced between metal bioaccumulation and histopathological indices in European hake, although the biometric parameters showed a relation with metal bioaccumulation (except Zn). In the second PCA associated with SQG-Qs, no correlation between histopathological lesions and SQG-Qs was evidenced. A comparison of the PCA analysis based on each histopathological index showed a higher correlation between both organs according to Lang et al. (2006) than according to Costa et al. (2009).

4. Discussion

Estimated metal SQG-Qs in the Basque continental shelf were classified as moderately impacted and presented similar values to those reported by Fonseca et al. (2013) in Mira, which is considered to be a non-polluted site, and lower values than those found for highly polluted estuaries (Carreira et al., 2013; Martins et al., 2012). According to the organic SQG-Qs, the sediments of the Basque continental shelf present no potential biological risk since they are classified as non-impacted. Although significant differences were found for Zn and organic compounds between the West and East, the potential risk of sediments causing biological adverse effects remained similar along the Basque continental shelf. Thus, metal bioaccumulation in liver showed similar values in common sole from the West and East and, in general, the metal content in liver was lower than the levels found in common sole from the NE Iberian Peninsula (Mhadhbi et al., 2012). In contrast, higher values than those recorded for juvenile common sole from estuaries in the South Iberian Peninsula were obtained (Usero et al., 2003). This higher metal bioaccumulation level in common sole from the Basque continental shelf could be attributed to the higher age of the fish (Magalhães et al., 2007). European hake presented similar metal bioaccumulation levels to those recorded in European hake from Scotland (Mormede and Davies, 2001). Although European hake occupies a higher trophic position than common sole within food webs (Sá et al., 2003; Sánchez and Gil, 2000), and therefore a higher bioaccumulation level is expected (van der Oost et al., 2003), common soles presented higher metal bioaccumulation levels than European hake partly due to their benthic behaviour (Morrison et al., 1997).

In light of the above, the use of common sole and European hake as sentinel organisms can be considered to be complementary, covering the biomonitoring of sediments and deep water areas in the Basque continental shelf. In general, the two histopathological indices calculated revealed no significant differences between common sole and European hake in either of the organs studied. However, common sole always presented more severe histopathological indices than European hake in liver, which could again be related to its benthic behaviour and high sensitivity (Costa et al., 2009; 2011).

The present findings do not reveal significant spatial differences in histopathological alterations of common sole from the West and East of the Basque continental shelf. This could be due to the lack of significant differences in metal sediment contamination levels or the fact that the levels were within the metal background range reported by

Legorburu et al. (2013) for the Basque continental shelf. Moreover, although significantly higher organic contamination levels were observed in the East than in the West, SQG-Qs indicated that sediments were non-impacted. The inter-campaign histopathological differences in both species were not associated with the variability of sediment contamination levels, thereby suggesting that other factors contribute to the histopathological assessment.

In agreement with this, one of the main drawbacks to using wild organisms in biomonitoring environmental pollution is the number of potential confounding factors that may mask the real histopathological damage (Au, 2004; Gonçalves et al., 2013; van der Oost et al., 2003). Thus, in this study we attempted to assess the age (by otoliths or well-defined length-age regressions), nutritional status (by calculating the condition factor), developmental stage (by gonad histology) and seasonality (by sampling in the same season) in order to minimise the natural variability of biological samples.

At a whole body level, externally visible diseases were rare in both species, which is in accordance with the previous finding that the Basque continental shelf is slightly impacted (Borja et al., 2011). Unlike in the current work, fin rot, ulcerous skin diseases and other alterations are pervasive in animals collected from notoriously impacted areas (Stentiford et al., 2009). However, pigmentation anomalies were observed in common sole from both sampling areas, although the prevalence of hyperpigmentation in this study was lower than that reported in dab from the North Sea but higher than in dab from the English Channel, Celtic Sea and Baltic Sea (Grütjen et al., 2013). Such hyperpigmentation may be linked to age, nutritional factors and/or effects of UV-B radiation on the early life stages of fish (Grütjen et al., 2013).

Liver was more sensitive than gonads as more histopathological lesions were recorded in both species. This is closely related to the biological role of the liver, which is the primary organ for detoxification of metal and organic xenobiotics in teleosts (Hinton et al., 2001). Most of the lesions recorded in the two species were considered to be non-specific alterations, thus indicating that liver function was not severely affected (ICES, 2004). The two histopathological index approaches tested herein showed consistent results as regards the prevalence of alterations in both species, thus indicating that the frequency and severity of each lesion were associated. The histopathological indices for gonad revealed no significant differences between species and gender, although male histopathological indices were always slightly lower than those of females, probably due to their reproductive role. A similar effect has been reported in some previous studies (Koehler, 2004), whereas others identified males as the most sensitive gender (Medina et al., 2012).

In histopathological terms, spongiosis hepatis was the most prevalent alteration in the liver of common sole. This alteration was first recorded in rats (Bannasch et al., 1981) and then in fish (medaka) exposed to methylazoxymethanol acetate during carcinogenesis studies (Hinton et al., 1984). Additionally, this alteration has been reported in fish exposed to chemicals in the laboratory and from contaminated regions (Agamy, 2012; Couch, 1991). It also seems to be positively correlated with age (Boorman et al., 1997; Ding et al., 2010). Thus, owing to the low sediment pollution levels found in the present study, age may be the main factor accounting for this lesion. Fat vacuolation of hepatocytes was observed to a higher degree in European hake than in common sole. In zebrafish, experimental exposure to organochemical pollutants, such as dioxins, provokes hepatic lipid accumulation (Zodrow et al., 2004), which can cause higher production of oxygen radicals, thereby provoking hepatic lesions (Vethaak and

Wester, 1996). Despite this, remarkable differences in the amount of accumulated fat can be found between presumably healthy specimens, especially if nutritional status, season and/or developmental stage are different (Domínguez-Petit et al., 2010). This could be confusing, especially in European hake, which naturally accumulate large amounts of lipids, a characteristic that is typical of gadiform fishes such as cod (*Gadus morhua*) or haddock (*Melanogrammus aeglefinus*) (Nanton, 2003). HNP are thought to be an alteration related to sub-lethally injured cells resulting from exposure to several types of toxicants (ICES, 2004; Myers et al., 1992). In contrast, the risk for developing nuclear pleomorphism increases with fish age (Stentiford et al., 2003). HNP prevalences in the present work were lower than those recorded by Marigómez et al. (2006) in the Basque continental shelf after the Prestige oil spill, which could suggest a recovery. The increase in MMCs has been related to a stress response possibly resulting from exposure to toxic compounds (Stentiford et al., 2003), but also increases with individual age and varies with the reproductive cycle (Vethaak and Wester, 1996). As both target species showed similar age and gonad development within the individuals sampled, these biological factors may not influence MMC and HNP frequencies.

No neoplasms, either benign or malignant, were found in either species in this study. Hepatic neoplasms are rare in young wild fish and do not appear with appreciable prevalence in free-living fishes until the age of 3 years (Myers et al., 1992). In common sole, despite the lack of differences in age and sediment pollution levels, necrosis, fat vacuolation and spongiosis hepatis showed significant differences between sampling area and campaigns, thereby suggesting that other factors are causative agents. In European hake, liver presented significant inter-campaign differences as regards haemorrhage, necrosis and concentric periductal fibrosis, which were consistent with the length of individuals. Thus, these statistical differences could be associated with age differences (Cachot et al., 2013; Myers et al., 1992). In general, those alterations recorded in the liver of both species presented background levels or were related with low pollution levels (Gonçalves et al., 2013; Marigómez et al., 2006).

Common sole and European hake of both genders mainly showed early gametogenic stages, which is in accordance with their reproductive cycle (Alvarez eta al., 2004; Ramos, 1982). Wild common sole from the Mediterranean Sea present a spawning period from January to March (Ramos, 1982), whereas the spawning of common sole is characterised by being asynchronous (Murua and Saborido-Rey, 2003), thus meaning that gametes of all stages of development are present, with no dominant population. European hake is a multiple egg batch-spawner (Murua and Motos, 2006) that spawns in the Bay of Biscay from January to May, with a defined spawning peak between February and March (Álvarez et al., 2004).

As expected, lower histopathological alterations were recorded in gonads than in liver. In common sole, atresia values were higher than those reported in the same species from the North and Irish Seas (Witthames and Greer Walker, 1995). A high prevalence of atretic oocytes in early reproductive stages has been related with exposure to contaminants (Reynolds et al., 2003). However, in order to decipher whether this alteration is related to contaminant levels, basal levels of atresia should be established in common sole from the Basque continental shelf. For European hake, the prevalence of atresia in oocytes in the primary growth stage was similar to that reported by Murua and Motos (2006), thus suggesting that these levels represent natural background levels. Moreover, histopathological assessment underlined the presence of lipid droplets in the oocytes of common soles. This accumulation of lipid in oocytes has been related to their energetic role within oocyte development (Sharma et al., 1996). The lack of intersex cases in the two species suggests low concentrations of endocrine disrupting chemicals.

With regard to the two histopathological indices applied, despite their application and development in different sentinel organisms (the histopathological indices proposed by Costa et al. (2009) in common sole and that proposed by Lang et al. (2006) in flounder), a high correlation was observed between them in both organs. Thus, the application of both approaches in diverse sentinel species can give a suitable histopathological assessment for biomonitoring of the health status of the marine environment. The histopathological indices estimated according to Costa et al. (2009) showed more sensitivity as regards understanding the mechanisms involved in tissue damage, together with the dissemination of each one, whereas only the most severe lesion and its dissemination are considered in the index proposed by Lang et al. (2006). Conversely, the rapid estimation of the histopathological indices and the non-dependence of the number of recorded histopathological biomarkers may facilitate data analysis.

5. Conclusions

Two ecological and commercial target species, namely common sole and European hake, seem to be good and complementary candidates as sentinel organisms for biomonitoring studies in the Basque continental shelf, although common sole manifested higher sensitivity to low environmental pollution. Additionally, this study has contributed to our understanding of the hepatic and gonadal histopathology of both sentinel species. Progressive changes in the liver, together with inflammatory responses, were the most notable reaction patterns. Gonads in the early stages of gametogenesis presented circulatory and inflammatory responses as the most frequent reaction patterns. The usefulness of developing single semi-quantitative histopathological indices to integrate all lesions, which allows a better understanding of environmental status, has been demonstrated. Both histopathological indices applied in this study have been shown to be accurate and solid tools for reflecting the health status of sentinel organisms. Thus, fish histopathology together with sediment and biota contamination levels could help to monitor compliance with the premises of Descriptor 8 in the MSFD. Despite this, further research is needed in order to adequately establish basal levels and potential confounding factors.

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Chapter 5

Histopathological baseline levels and confounding factors in common sole for marine environmental risk assessment

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Summary

Liver and gonad histopathology, biometric parameters and hepatic metal bioaccumulation were assessed monthly over a one-year period in common soles from the Basque continental shelf, in order to determine baseline levels and confounding factors within biomonitoring studies. Biometric parameters and hepatic metal bioaccumulation varied according to season and gender. Accordingly, hepatic histopathological traits presented seasonal variations related to the reproductive cycle. However, the hepatic histopathological index showed that seasonality and gender were not significant confounding factors. Conversely, the gonad histopathological index was modulated by season and gender. As for organ comparison, the liver endured more severe histopathological damage than the gonad. In brief, the sampling period and gender may not affect the estimation of hepatic histopathological indices for biomonitoring purposes. Nonetheless, due to different sensitivities to environmental 'noise' variables, the sampling period and gender differentiation should be thoroughly considered for the assessment of gonad histopathology, biometrics and metal bioaccumulation.

1. Introduction

European marine systems are valuable environmental heritages that must be protected, restored and preserved, since these coastal and offshore waters are often affected by human activities that may cause environmental degradation (Borja et al., 2010; Davies and Vethaak, 2012; Gago et al., 2014; Meybeck and Helmer 1996). For this reason, environmental policies and guidelines are being drawn up for the protection of marine environments which include the overall management of contaminant inputs (Borja et al., 2008). With this goal in mind, the European MSFD (Directive 2008/56/EC) recently established eleven Descriptors that need to be met in order to achieve GES of the marine environment by 2020. Among these qualitative criteria, Descriptor 8 is described as “Concentrations of contaminants are at levels not giving rise to pollution effects”. Thus, achieving GES relies on the assessment of contaminant levels at different matrices, their adverse effects on aquatic organisms and their causal relationship (Borja et al., 2010; Gago et al., 2014; Lyons et al., 2010). Likewise, in order to attain the premise in Descriptor 8, fish diseases and histopathological alterations have been used as indicators of pollution effects as they provide a relevant individual biological end-point of historical exposure (*e.g.* Cuevas et al., 2015; Feist et al., 2015; Gonçalves et al., 2013).

The use of fish as sentinel organisms for the biomonitoring of pollutant-induced adverse biological effects is long believed to be of importance due to their ecological and economical relevance and even their physiological similarity to other vertebrates (Bolis et al., 2001; Gago et al., 2014). As it has been abovementioned (Chapter 4), flatfishes, especially the common sole (*S. solea*) and the Senegalese sole (*S. senegalensis*), have been widely pointed out in ecotoxicological studies carried out in estuarine, coastal and marine environments of S Europe (*e.g.* Costa et al., 2009; Cuevas et al., 2015; Fonseca et al., 2011; Riba et al., 2004; Siscar et al., 2013).

Nevertheless, for the implementation of histopathology in biomonitoring studies, the determination of histopathological baseline levels in wild common soles is still required in order to enable differentiation between pollution-induced effects and basal physiological responses (Brenner et al., 2014; Fricke et al., 2012; Schmidt et al., 2013). The term 'baseline level' has several definitions in environmental studies, although it is generally used to refer to the natural levels of biological responses of organisms from pristine or negligibly disturbed areas (Borja et al., 2010). However, one of the problems in deriving reference conditions arises from the absence of non-impacted areas holding the same biogeographical parameters as impacted sites. Nevertheless, the Basque continental shelf has been considered as a pristine area within the SE Bay of Biscay due to its offshore water conditions and low human pressures, which may contribute to its qualification as a reference area within ICES subarea VIII (see Borja et al., 2011; Cuevas et al., 2015).

Furthermore, the characterisation of confounding factors is pivotal for the implementation of histopathology in biomonitoring approaches using common soles (Gonçalves et al., 2013). In fact, one of the main drawbacks of surveying wild organisms in marine environmental risk assessment is the relatively high number of potential biological and environmental confounding factors that may hamper the evaluation of biological responses to pollutants (Bignell et al., 2008; Bocchetti et al., 2008; Brenner et al., 2014; Garmendia et al., 2010; Izagirre et al., 2014).

As a contribution to the establishment of histopathological indices in fish for biomonitoring within the scope of the MSFD, the main objectives of the present work were: (*1*) to determine baseline levels of hepatic and gonadal histopathological traits and relate these to hepatic metal bioaccumulation levels in common sole (*S. solea*) collected from the Basque continental shelf, presumably a pristine area; and (*2*) to establish how confounding factors, such as seasonality, age and gender, may influence the histopathological assessment and subsequent data interpretation.

2. Material and methods

2.1. Sampling area and sample collection

Adult common sole (n = 30, total of 300) were collected monthly with a trammel net from June 2012 to May 2013 at a 50 m depth along the Basque Continental Shelf (SE Bay of Biscay) (Figure 5.1). The Basque continental shelf, owing to its offshore waters conditions has been considered a pristine or minor disturbed area, although some pressures such as a gas storage platform, four disposal sites for dredged material and fisheries have been described (Borja et al., 2011).

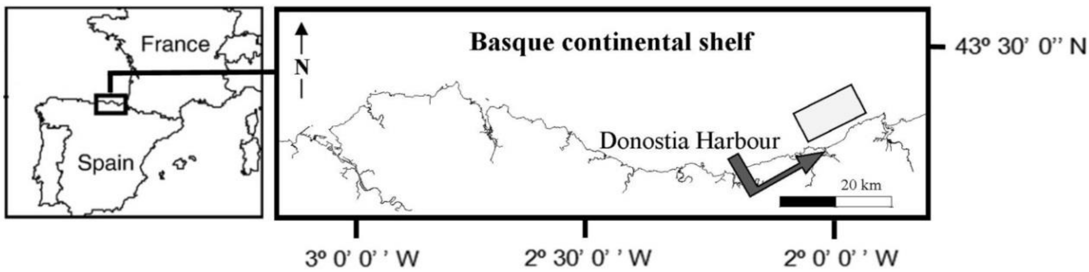

Figure 5.1. Map of the study area, located in the Basque continental shelf (SE Bay of Biscay), showing the fishing area (grey square) and the Donostia Harbour.

Sample collection was not feasible in October, while half of the sample (n ≈ 15) was only obtained in September 2012 and May 2013. The animals were transported on ice to the laboratory (≈ 4 h after sample landing off at Donostia, see Figure 5.1), where the total length (cm) and weight (g), weight (g) of the liver and gonad, the gender and gross pathological traits (through the presence of externally visible diseases) were recorded for each individual using the methodology proposed by ICES (1996). Afterwards, multiple liver samples were collected and frozen at -20 ºC for bioaccumulation analyses or fixed in Davidson’s fluid (10% formalin, 10% acetic acid and 30% ethanol) for 48 hrs. Gonads were also fixed in 10% neutral-phosphate buffered formalin for 18 – 24 hrs for histological evaluation.

2.2. Biometric parameters and externally visible diseases

Three biological indices were calculated per individual from the previously recorded biometric data as follows: (*1*) the condition factor (K = (total weight (g)/length (cm)^3× 100); (*2*) the hepatosomatic index (HSI = (liver weight (g)/body weight (g) × 100) and (*3*) the gonadosomatic index (GSI = gonad weight (g)/body weight (g) × 100). Age was also determined from *sagittae* otoliths. The prevalence of externally visible diseases, (% = (number of cases/total cases analysed) × 100), was estimated per month.

2.3. Metal bioaccumulation levels

Metals (Cd, Cu, Hg, Pb and Zn) were measured (mg kg^{-1} in wet weight) in two pools of livers per gender and month of collection. Liver samples were homogenised and digested in 10 ml of concentrated HNO_3 in a microwave oven (MARS 5 Xpress CEM Corporation Instrument). Metal content was then determined by AAS (using an AAS800 model apparatus, Perkin Elmer). Total mercury was determined using the cold vapour technique and the remaining metals (Cd, Cu, Pb and Zn) by graphite furnace equipped with a Zeeman background correction device. The validation of the analytical procedure was carried out using dogfish muscle as certified reference material (DORM-2 from NRC), with the results being found within the certified range. The mean recoveries were in the range of 80 – 90% for all of the metals, throughout the study.

2.4. Histology and histopathological procedure

Fixed liver and gonad portions were embedded in glycol methacrylate resin (Technovit 7100, Heraeus Kulzer) and sections of 3 – 5 µm thick were obtained using a Microm HM 350 S rotary microtome. Sections were subsequently stained with H&E and examined with an Olympus BX60 light microscope. The accuracy of histopathological analyses was tested by blind reviews.

In order to identify the main alterations in both target organs, the prevalence of histopathological lesions, (% = (number of cases/total cases analysed) × 100), was estimated per month for the liver and per month and gender for the gonad. Additionally, gonad development was determined according to García-López et al. (2006) for males and as proposed by Murua and Motos (2006) for females.

A semi-quantitative histopathological approach based on the weighted histopathological indices developed by Bernet et al. (1999) and adapted by Costa et al. (2009) was determined for the liver and gonad. The hitherto proposed histopathological index considers the relative biological importance (weight) and the dissemination degree (score) of each lesion per organ. The histopathological lesions were classified into four reaction patterns: (*1*) circulatory disturbances; (*2*) inflammatory responses; (*3*) regressive changes (implying functional loss) and (*4*) progressive changes (involving altered function). The global histopathological indices (*Ih*) were calculated for each individual and organ as:

$$Ih = \sum_{1}^{j} w_j a_{jh}$$

Where w_j is the weight of the j histopathological trait and a_j the score for the j alteration of the h individual. The weights ranged from 1 to 3 (from minor, as hyperaemia, to high severity, such as necrosis), while the score ranged from 0 (feature or alteration not observed) to 6 (diffuse). In Table 5.1, the main histopathological alterations in the liver and gonad of common soles and their respective biological importance (weight) are summarised.

Table 5.1. The main histopathological alterations classified by the reaction pattern in the liver and gonad of common soles collected in the Basque continental shelf and their respective weights (w). MMCs: melanomacrophage centres; HNP: hepatocellular and nuclear pleomorphism; HV: hydropic vacuolation.

Reaction pattern	Liver alteration	w	Gonad alteration	w
Circulatory disturbances	Hyperaemia	1	Hyperaemia	1
	Haemorrhage	1		
Inflammatory responses	MMCs	1	MMCs	1
	Lymphocytic infiltration	2	Granulomatosis	2
			Lymphocytic infiltration	2
Regressive changes	HNP	2	Necrosis	3
	Necrosis	3	Pyknotic oocytes/ spermatocytes	2
			Atresia /Intersex	3
Progressive changes	Fat vacuolation of hepatocytes	1		
	Spongiosis hepatis	2		
	Concentric periductal fibrosis of bile duct	2		
	HV of epithelial cells of bile ducts	2		

2.5. Statistical analyses

Statistical analyses were performed with Statgraphics Plus 5.0 software. Normality and homoscedasticity of the data was checked by the Shapiro-Wilk's and Levene's tests, respectively. The non-parametric Kruskal-Wallis One-way ANOVA by ranks was used to analyse differences in biometric and histopathological data of common soles. Biometric data was analysed per month and gender, while histopathological results were tested per month to determine seasonal fluctuations and, globally and/or per month to assess significant differences between genders and ages. The non-parametric Spearman's rank-order R statistic was used to assess correlations between biometric parameters, histopathological indices and metal bioaccumulation. Standardised major axis regression analyses were carried out to evaluate the relationship between metal bioaccumulation levels in males and females collected over one year. Slope and intercept were tested to be significantly different from 1 and 0, respectively, according to Warton et al. (2006). These latter analyses were performed using the SMATR software (Falster et al., 2006). All analyses were carried out at $p < 0.05$ significance level.

3. Results

3.1. Biometric parameters and externally visible diseases

The mean ± standard deviation of total length, total weight and K of common soles collected along the Basque continental shelf over one year were 33.7 ± 4.3 cm, 385 ± 171 g and 1.0 ± 0.1 g cm^{-3}, respectively (Figure 5.2). In females, a significant increase in length and weight was observed between January and February, whereas in males length and weight remained fairly constant throughout the one-year cycle. In general, females were larger than males, especially from November to April (Figure 5.2A and 5.2B). The K, however, did not vary throughout the one-year cycle for either gender, although it was significantly higher for females than for males in December and February (Figure 5.2C). The age of common soles ranged between 2 to 5 years (Figure 5.2D).

HSI and GSI biological indices per month and gender are shown in Figure 5.3. In females, HSI ranged between 0.52 to 0.64 g g^{-1} from March to November, but in December and February two main peaks (0.99 and 1.12 g g^{-1} respectively) were registered. In males, however, HSI was relatively steady (0.52 – 0.64 g g^{-1}) throughout a one-year cycle. Moreover, HSI values in August, November, December, February and

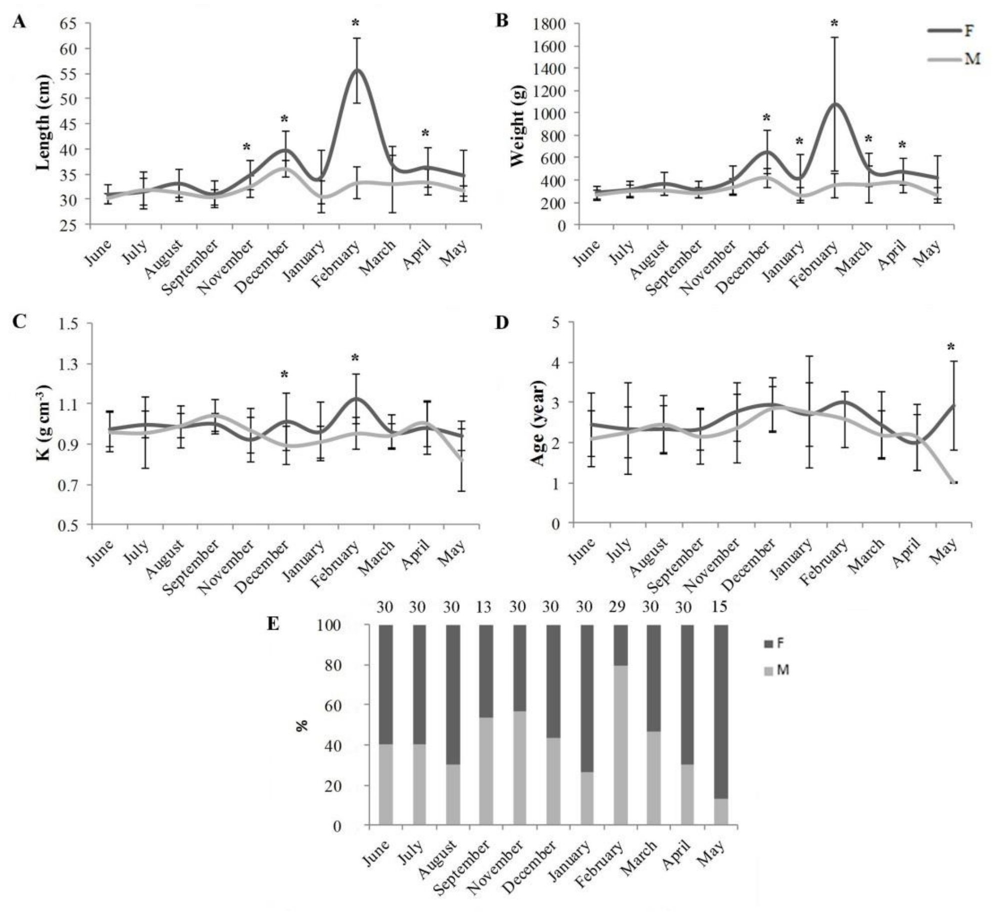

Figure 5.2. Seasonal variation of (*A*) length, (*B*) weight, (*C*) K and (*D*) age (mean ± standard deviation) per gender, together with (*E*) sex ratio of common soles collected along the Basque continental shelf. * indicates statistical significant differences ($p < 0.05$) between genders per month. Sample size per month is indicated above each column. F: female, M: male.

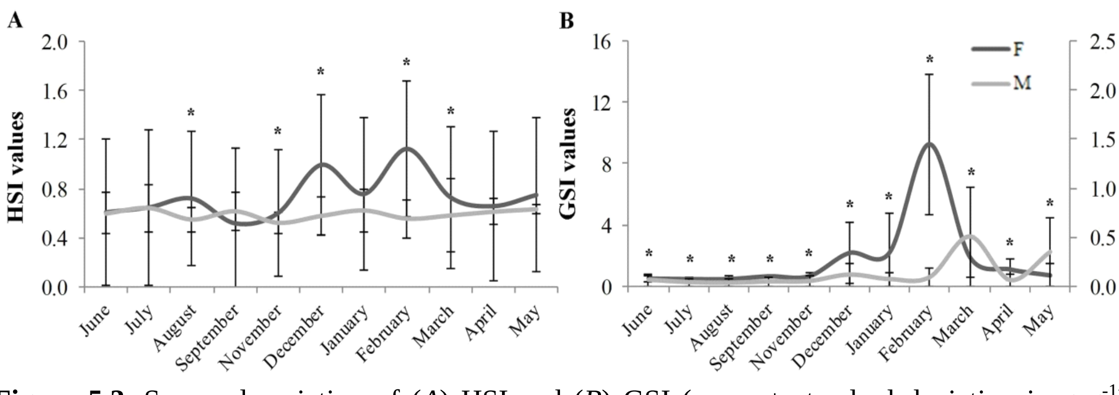

Figure 5.3. Seasonal variation of (*A*) HSI and (*B*) GSI (mean ± standard deviation in g g^{-1}) represented by gender of common soles collected along the Basque continental shelf. * indicates statistical significant differences ($p < 0.05$) between genders per month. Left Y axis: females (F); right Y axis: males (M).

March were significantly higher in females than in males (Figure 5.3A). On the other hand, GSI values for females were consistently higher than in males, ranging between 0.45 to 1.89 g g^{-1} from March to November and between 2.16 – 9.27 g g^{-1} in the remaining months, with one main peak (9.27 g g^{-1}) in February. In males, GSI was fairly constant (0.05 – 0.35 g g^{-1}) throughout the year, as occurred for HSI (Figure 5.3B).

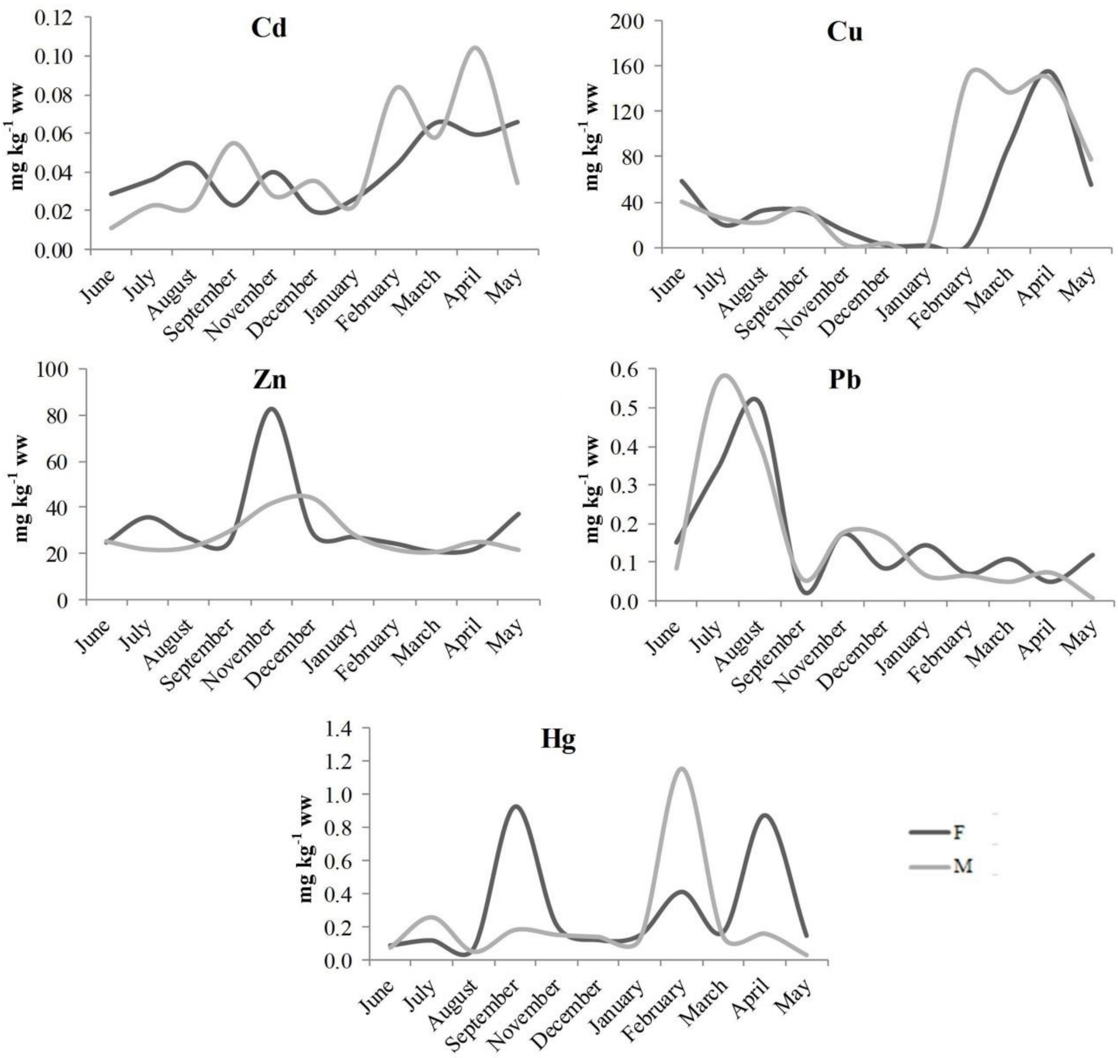

Figure 5.4. Seasonal variation of hepatic metal bioaccumulation levels (mg kg^{-1} ww) represented by gender in common soles collected along the Basque continental shelf. F: female, M: male.

Most externally visible diseases recorded during a one-year cycle related to skin pigmentation, either as hyperpigmentation (6 – 53%) on the blind side or as depigmentation on the ocular side (3 – 13%). The frequency of spinal deformation was also recorded in a few individuals (< 3%). No significant signs of gross external or internal pathological traits, such as infections, ulcers or tumours were observed.

3.2. Metal bioaccumulation levels

Hepatic metal bioaccumulation levels are illustrated in Figure 5.4. Cadmium and Cu levels presented a similar seasonal pattern, with highest values from January to March. Zinc also yielded a seasonal variation with a main peak in November. According to the standardised major axis regression analyses, Zn was the only metal that differed ($p < 0.01$) from 1:1 in the relationship between genders, showing significantly higher bioaccumulation levels in females than in males. Lead showed relatively constant bioaccumulation levels over a year, except for a peak in July for both genders. Finally, Hg did not show any specific seasonal pattern, presenting two peaks in September and April for females, with one in February for males.

3.3. Histopathological analyses

3.3.1. Histopathological description and prevalence in the liver

The normal liver of common soles presented hepatocytes adjacent to sinusoids branching out of a blood vessel (Figure 5.5A). The main circulatory disturbances were hyperaemia and haemorrhage. Individuals revealing these two alterations were more frequent in September and from November to February, although haemorrhage was less frequent than hyperaemia (Figure 5.6A). Inflammatory responses, recorded as MMCs and lymphocytic infiltration (Figure 5.5C), were similarly prevalent from June to November, but MMCs increased consistently over the remaining months while lymphocytic infiltration decreased (Figure 5.6B).

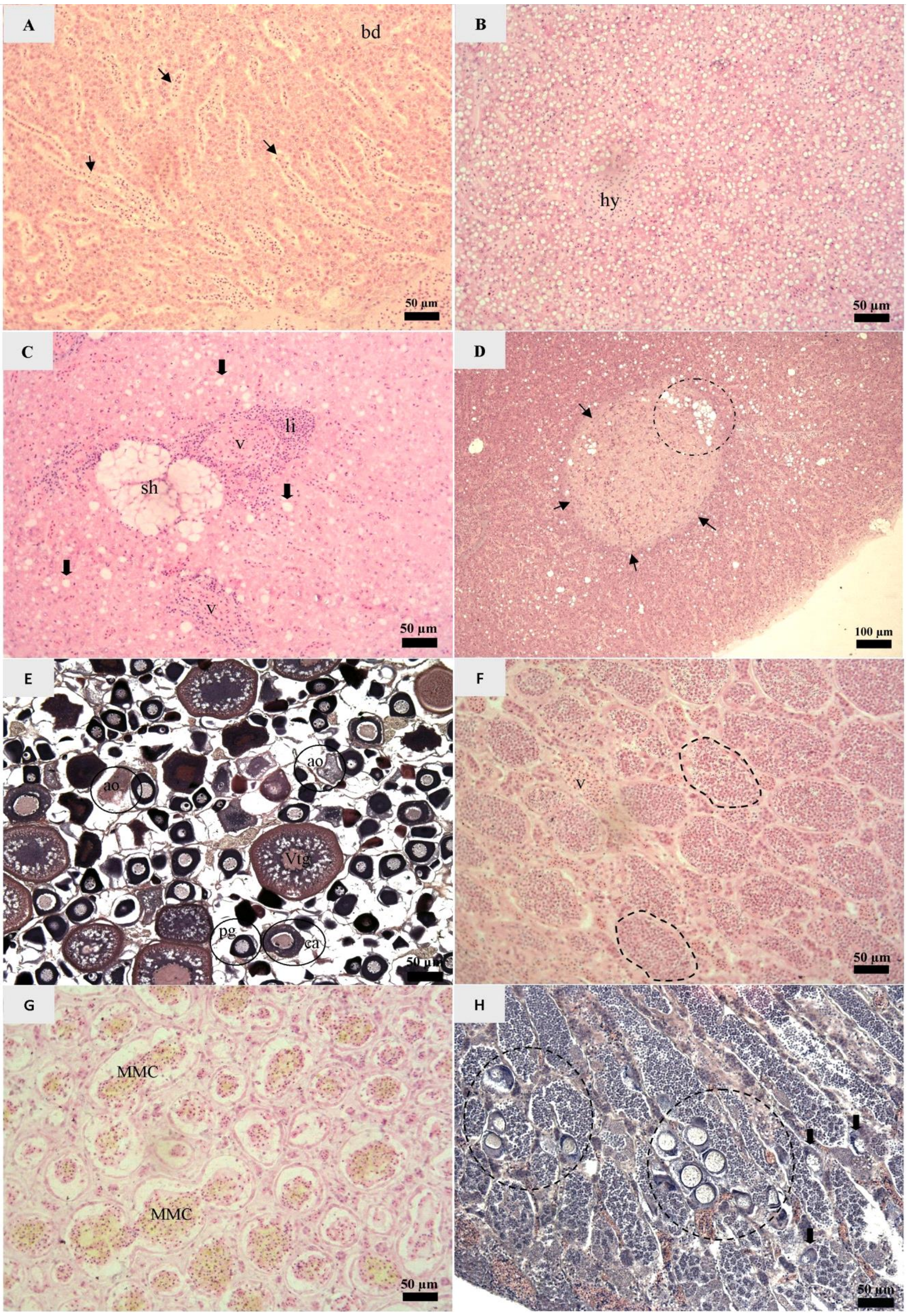

Figure 5.5. Liver and gonad sections of common soles collected along the Basque continental shelf stained with haematoxylin-eosin (H&E). (*A*) Normal hepatic tissue with hepatocytes aligned with sinusoids (arrows), showing a normal bile duct (bd) (*B*) Hepatic tissue homogeneously affected by mild fat vacuolation of hepatocytes, showing a hyperaemia (hy). (*C*) Spongiosis hepatis (sh) and lymphocytic infiltration (li) close to a blood vessel (v) in the hepatic tissue surrounded by fat vacuolation of hepatocytes (arrows). (*D*) An adenoma (arrows) in the hepatic tissue surrounded by focal spongiosis hepatis (dashed circle). (*E*) Normal female gonadal tissue showing oocytes in primary growth (pg), cortical alveoli (ca) and early vitellogenic stage (Vtg), together with some atretic oocytes (ao). (*F*) Normal male gonad presenting germinal follicles (dashed circle) in mid spermatogenesis stage. (*G*) Male gonad tissue in resting stage manifesting melanomacrophage centres (MMC) with erythrocytes within germinal follicles. (*H*) Intersex condition in male gonadal tissue showing oocytes in primary growth (dashed circles and arrows). v: blood vessel.

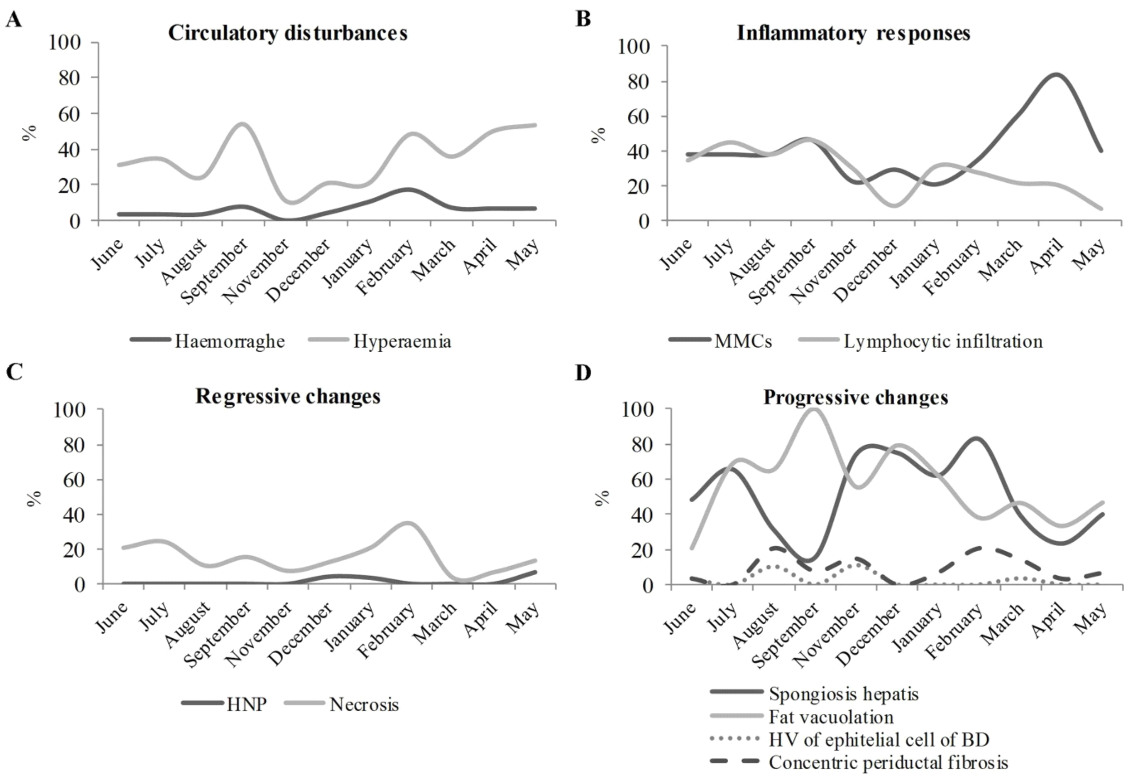

Figure 5.6. Seasonal variation in the prevalence of histopathological alterations recorded in the liver of common soles collected along the Basque continental shelf classified per reaction pattern. (*A*) Circulatory disturbances, (*B*) Inflammatory responses, (*C*) Regressive changes and (*D*) Progressive changes. HV: hydropic vacuolation; BD: bile duct; HNP: Hepatocellular and nuclear pleomorphism; MMCs: melanomacrophage centres.

The main regressive changes were HNP of hepatocytes and necrosis. Both alterations presented fairly low prevalence, except for a higher necrosis prevalence peak observed in February (Figure 5.6C). Fat vacuolation and spongiosis hepatis were the most frequent progressive alterations recorded in the liver of common soles (Figures 5.5B and 5.5C). Fat vacuolation reached its maximum prevalence from July to December; although it decreased after February. Spongiosis hepatis, however, was most frequent from November to February (Figure 5.6D). Hydropic vacuolation of epithelial cells and concentric periductal fibrosis of bile ducts showed lower and relatively steady prevalence throughout the year cycle (Figure 5.6D). Only one case of a benign tumour (adenoma) was observed in a female common sole collected in July (Figure 5.5D), although no malignancies were recorded.

3.3.2. Histopathological description and prevalence in the gonad

The reproductive cycle in both genders of common soles showed an asynchronous gamete development and batch spawner pattern (Figure 5.7). In general, early gametogenesis occurred in both genders from May to August presenting mainly primary growth oocytes and spermatogonia within germinal follicles. Subsequently, mid-to-late gametogenesis occurred from September to November characterised by vitellogenic oocytes and primary and secondary spermatocytes (Figures 5.5E, 5.5F and 5.7). The spawning period lasted from December to March showing germinal follicles full of mature oocytes and spermatozoa, while the recovery of gonads took place between March and April.

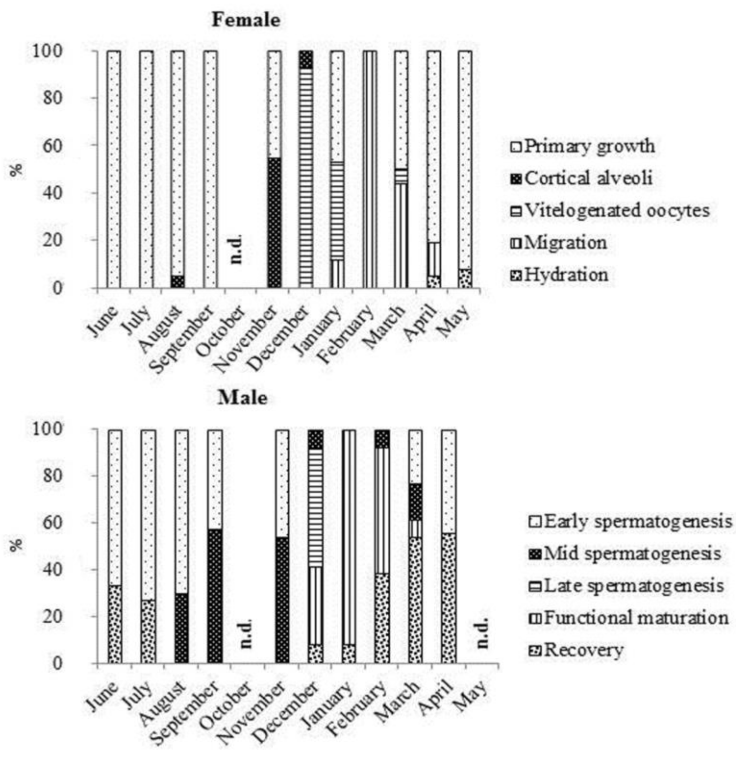

Figure 5.7. Gamete developmental stages of female and male common soles collected along the Basque continental shelf over the one-year cycle. n.d.: no data.

Hyperaemia, MMCs and some isolated cases of lymphocytic infiltration and granulomatosis fluctuated without a well-defined pattern during one year in both genders (Figures 5.5G, 5.8A and 5.8C). Nevertheless, these alterations were most frequent during the spawning period, from December to March. In females, pyknotic, necrotic and atretic oocytes were shown, with atresia the most prevalent alteration especially during the spawning and early post-spawning period (Figure 5.8B). Pycnosis and necrosis of oocytes, however, were recorded in fairly low prevalence. In males, necrosis and intersex were scarce (Figure 5.5H). Regressive alterations were more frequent in females than in males (Figures 5.8B and 5.8D). No progressive lesions were recorded in both genders.

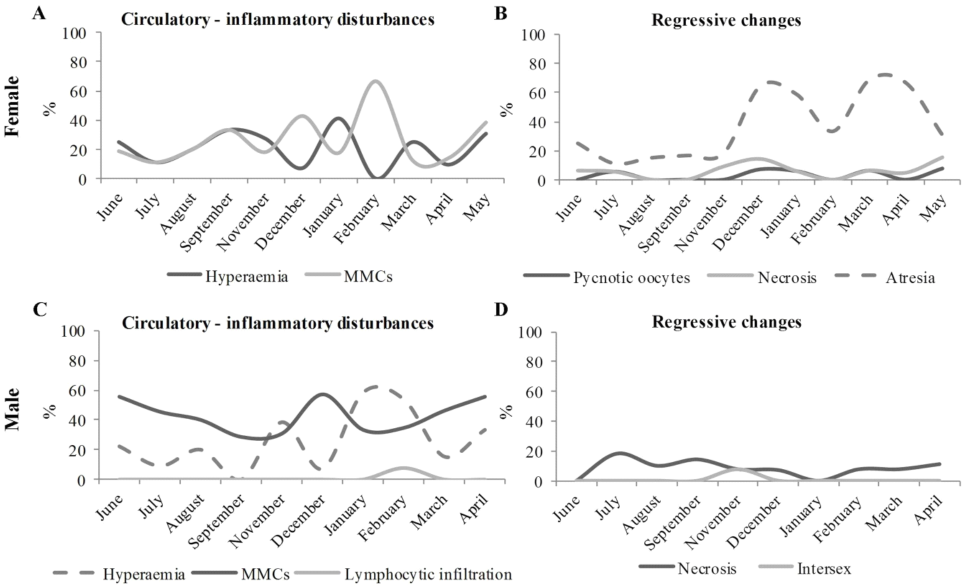

Figure 5.8. Seasonal variation in the prevalence of histopathological alterations recorded in the gonad of common soles collected along the Basque continental shelf classified per reaction pattern and gender. Circulatory-inflammatory disturbances and regressive changes (*A* and *B*) in females and (*C* and *D*) in males. MMCs: melanomacrophage centres.

3.3.3. Histopathological indices of the liver and gonad

The hepatic histopathological index did not significantly vary over the one-year cycle for either gender, ranging from 7.5 to 10.1 in females and from 7.0 to 12.8 in males (Figure 5.9A), albeit without significant differences between genders (Figure 5.10A). Regarding age, no significant differences were registered in the hepatic histopathological index (Figure 5.10C), although progressive changes were more frequent in older individuals than in younger ones.

Gonad histopathological index was higher in females than in males (Figure 5.10B), being significant in December, January and March (Figures 5.9B and 5.10B). Females presented significantly higher values during the spawning period from December to March (3.3 – 6.3) than in the rest of the months. Male histopathological index, however, remained steady over a year (1.3 – 3.1) (Fig. 9B). No significant differences in gonad histopathological index values were shown between common soles of different ages (Figure 5.10D).

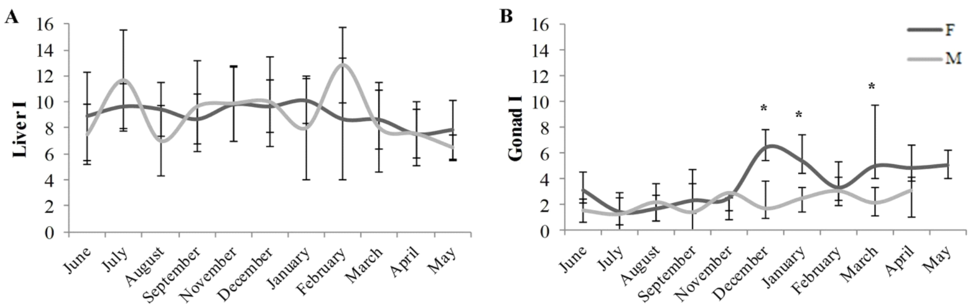

Figure 5.9. Seasonal variation of mean histopathological indices of the (*A*) liver and (*B*) gonad of common soles collected along the Basque continental shelf by gender. Error bars represent 95% confidence intervals and * indicates significant differences ($p < 0.05$) between genders per month. F: female, M: male.

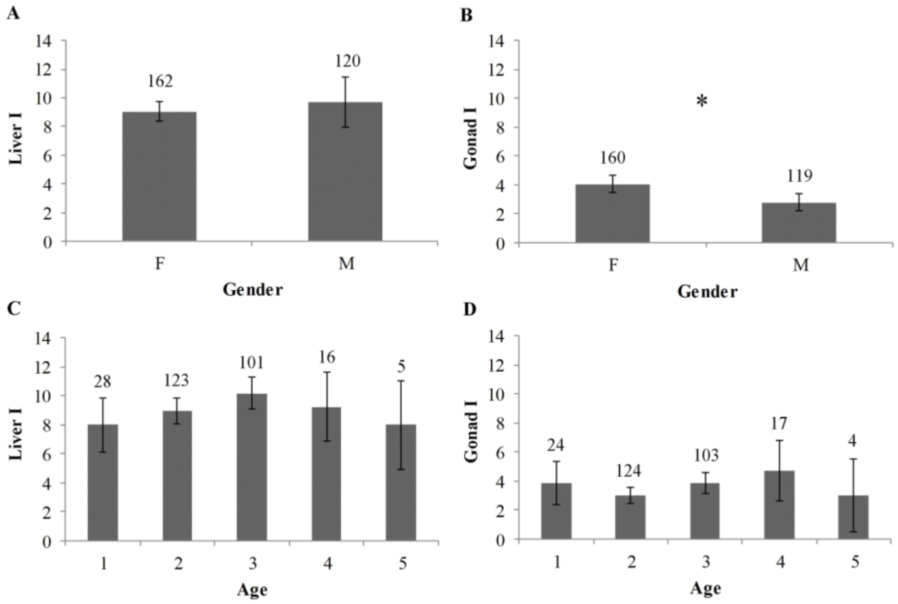

Figure 5.10. Mean histopathological indices of the liver and gonad of common soles collected along the Basque continental shelf represented by gender (*A* and *B*) and age (*C* and *D*). Error bars represent 95% confidence intervals, * indicates significant differences ($p < 0.05$) and the sample size of each experimental group is shown above each column. F: female, M: male.

3.3.4. Data integration

According to correlation analyses, Cd bioaccumulation levels in the liver were positively and significantly correlated with the circulatory disturbances condition index (R = 0.62, $p < 0.05$, n = 11) and negatively with the progressive changes condition index (R = -0.63, $p < 0.05$, n = 11). Similarly, hepatic Cu levels were highly correlated with the circulatory disturbances index (R = 0.81, $p < 0.05$, n = 11). However, there was not significant correlation between metals and liver histopathological index values. Furthermore, no significant correlations were recorded between the liver histopathological index and biometric parameters, or between the liver histopathological index and HSI. No significant correlations were observed between the gonad histopathological index and metal bioaccumulation levels in the liver. Accordingly, lack of correlation was shown either between the gonad histopathological index and GSI, or between the gonad histopathological index and biometric parameters. On the other hand, Cd levels in the liver were positively correlated with Cu (R = 0.80, $p < 0.05$, n = 11), Hg (R = 0.69, $p < 0.05$, n = 11) and Zn (R = 0.74, $p < 0.05$, n = 11) bioaccumulation levels.

4. Discussion

The present study describes the histopathological traits that may commonly be found in the liver and gonad, together with biometric parameters and hepatic metal bioaccumulation levels in common soles collected over a one-year cycle along the Basque continental shelf, presumably a pristine area. These findings thereby constitute a relevant set of baseline histopathological levels and confounding factors to be considered for biomonitoring the biological effects of pollutants using common soles as sentinel organisms, at least from similar biogeographical characteristics (*e.g.* ICES subarea VIII).

The characterisation of gonad development in sentinel organisms has been considered crucial within biomonitoring approaches in order to determine the influence of the reproductive cycle on histopathological assessment (Davies and Vethaak, 2012; Fricke et al., 2012). The reproductive cycle of common soles of either gender collected within this work started (evidenced by early gametogenesis) between May and August. Gametogenesis progressed (mid-late gametogenesis) during autumn until reaching mature gonads (spawning) from December to March, while the recovery phase (post-spawning) occurred in later winter and early spring. These findings are consistent with those recorded in soles collected along the Portuguese coast (Texeira and Cabral, 2009).

Fluctuations in the reproductive cycle and gender were reflected in the seasonal variation of biometric parameters and biological indices (HSI and GSI) of common soles. Hence, a maximum peak in length and weight was recorded at the start of the spawning period while a decreasing trend was registered in females after spawning. In contrast, length and weight variations in males were less distinct and remained steady throughout the annual cycle. This seasonal pattern may be related to the fishing effort shifting to areas where common soles were relatively smaller towards the end of the spawning, or to seasonal feeding and spawning migrations of common soles. In agreement, these variations in biometric parameters have been related with the migratory behaviour in soles from the North Sea (van Beek, 1988). Similarly, HSI and GSI increased when common soles showed gonads in advanced gametogenesis and spawning; while both biological indices decreased in post-spawning. This seasonal fluctuation of biological indices suggested that energy resources were allocated to reproduction during spawning, while during the rest of the year they were assigned to growth and fat deposition, as it has already been stated by Nunes et al.(2011) in the Atlantic sardine. On the contrary, the K of common soles collected within this study remained fairly constant over a one-year cycle, which, in agreement with Stevenson and Woods (2006), suggested a similar nutritional and health status of common soles. Hence, the seasonal variability of HSI, GSI and K was more pronounced and presented higher values in females than in males, especially at spawning period. This is consistent again with the aforementioned findings that females were involved more intensively in the reproductive cycle than males.

Conversely, most externally visible diseases were related to skin pigmentation and yielded an unclear relation to known environmental and gender-specific variation. It must be emphasised that the causes of skin pigmentation anomalies are still unknown, but there are indications that the condition may be linked to nutritional factors and/or effects of UV-B radiation during the early life stages of fish (Grütjen et al., 2013). The present findings suggest that these pathological traits are common in the species and that natural variation should not be excluded, which mandates caution when interpreting these alterations as potential biomarkers.

Bioaccumulation levels of almost all metals (Cd, Cu, Pb and Zn) in common soles also presented seasonal fluctuations during a one-year cycle, except Hg. Copper and Cd presented highly correlated bioaccumulation levels with highest values during the spawning, while Pb and Zn maximum levels were registered in early gametogenesis and pre-spawning, respectively. The seasonal pattern of Cu bioaccumulation, as an essential metal, may be related to the undisclosed natural requirements of this micronutrient (Grosell et al., 2007; Vasconcelos et al., 2011). On its turn, the peak in Zn bioaccumulation levels in pre-spawning females (not observed in males, Figure 5.4) may be associated with the increased activity of the liver during vitellogenesis (Olsson et al., 1989). Conversely, Cd, Hg and Pb bioaccumulation levels may be linked with different feeding habits of common soles and/or the bioavailability of these non-essential metals within the marine environment. Overall, the common soles collected along the Basque continental shelf presented lower metal bioaccumulation levels in the liver than soles from polluted areas (see for instance Fernandes et al., 2008; Mhadhbi et al., 2012; Oliva et al., 2013; Usero et al., 2003), sustaining the notion that the area may be regarded as a reference location.

Among liver histopathological lesions, fat vacuolation of hepatocytes and spongiosis hepatis were the most prevalent alterations in common soles collected along the Basque continental shelf. As occurred in this study, the degree of fat vacuolation of hepatocytes is highly dependent on the reproductive cycle, showing a seasonal pattern with the highest frequencies before vitellogenesis followed by a subsequent decrease (Davies and Vethaak, 2012; ICES, 2004). Spongiosis hepatis, however, presented the highest prevalence during early gametogenesis and spawning and higher values were recorded in older specimens. In agreement with this study, several research works stated that spongiosis hepatis was positively correlated with age (Boorman et al., 1997; Ding et al., 2010; Lang et al., 2006), being consequently a confounding factor that should be regarded on histopathological assessment and subsequent data interpretation of this lesion. The presence of necrosis and MMCs was only moderate and showed a mild peak of frequency in February, when fish presented gonads in last spawning and early resting stages. As expected, the occurrence of necrotic foci and defence cells was found to occur in parallel (*e.g.* Fricke et al., 2012). Thus, higher frequencies of MMCs were in accordance with the natural regeneration or reabsorption activities of the hepatic parenchyma related to the reproductive cycle (Koehler, 2004; Shulman and Love, 1999; Takashima and Hibiya, 1995). The frequency of other histopathological features fluctuated without a clear pattern over a one-year cycle, indicating that other natural biological and/or environmental factors may be responsible for the observed biological effects, such as natural variations in environmental elements. This idea is reinforced by the positive correlation found between Cd and Cu bioaccumulation levels and the circulatory disturbances condition index. Although the occurrence of most histopathological traits in the liver fluctuated during the one-year cycle, when the histopathological alterations were integrated into a single index, this index presented fairly steady values. Furthermore, no significant differences were registered between genders. Altogether, this significant lack of natural variation when integrating multiple histopathological traits may be a very significant asset of the approach for the purpose of biomonitoring with flatfish since the sampling period may be disregarded.

The current results indicate that the selection of a target organ is a critical issue in histopathological assessment. As expected, the liver endured more severe histopathological damage than the gonad, probably due to its detoxification role in teleosts. Therefore, the liver is recommended as the main target organ for histopathological assessment not only using common soles as sentinels but also for other fish species (Fernandes et al., 2008; Fricke et al., 2012; Hinton et al., 2001; Lang et al., 2006). Conversely, gonads yielded significantly higher histopathological indices in females than in males, especially during the spawning period. Histopathological indices in the male gonad, however, remained relatively steady and low throughout the year. This could be explained by the fact that the morphological and physiological changes suffered by females are more pronounced, due to the high metabolic and energy requirements of oogenesis, as already pointed out by other authors (*e.g.* Koehler, 2004; Koehler et al., 2004; Winzer et al., 2001). Thus, since seasonality and gender may act as confounding factors in gonad histopathology, the importance of the sampling period should not be neglected in sampling design and subsequent data interpretation. In this respect, the most suitable period to assess gonad histopathology in common soles is likely to be during the early-to-mid gametogenic stages, from June to September.

5. Conclusions

According to the present outcomes, biometric parameters, biological indices (HSI and GSI) and gonad histopathological indices varied according to reproductive cycle and gender. Zinc bioaccumulation levels presented a main peak in pre-spawning females, while Cd, Cu, and Pb demonstrated a seasonal bioaccumulation pattern in both genders, which mandates caution when interpreting these findings without considering natural biological and environmental variations. In turn, the prevalence of individual hepatic histopathological alterations showed seasonal changes influenced by the reproductive cycle. However, when all the alterations were integrated into the liver histopathological index, seasonality and gender did not act as confounding factors. This finding indicates that seasonality may not be critical when addressing hepatic histopathology in common soles through integrative multi-trait approaches, at least in animals collected from similar environments, unlike gonads. This important information should thus be integrated in biomonitoring procedures under the scope of Descriptor 8 of the MSFD, for the assessment of biological effects of pollutants using common soles as sentinel organisms.

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General discussion

As a result of the implementation of the WFD and MSFD to promote sustainable use of the seas and conserve marine ecosystems (*e.*g. living aquatic life, habitats and resources), it is crucial to develop not only chemical tools, but also biological effects measures for the determination of aquatic health status within an integrated assessment approach (Allan et al., 2006; Borja et al., 2010; Lehtonen et al., 2014; Zampoukas et al., 2012). In this respect, the present thesis follows the scheme of the integrated assessment of environmental pollution proposed by SGIMC (Davies and Vethaak, 2012), which combines biological effects methods in diverse sentinel organisms with sediment and tissue chemistry measurements (Figure D.1). This scheme has been modified to be applied within the Basque coast and it should be used in a tiered or decision tree approach. As such, depending on the objective of the study and the area to be investigated (*i.e.* estuarine, coastal or offshore environment), different sentinel organisms and different methodologies should be selected for the integrated assessment of environmental pollution (Figure D.1). For instance, if the aim of the study is to evaluate the effect of the use of TBT-based antifouling paints in a harbour, the most appropriate option would be to select gastropods as sentinel organisms and determine imposex levels and organotin tissue chemistry as specific responses to TBT exposure (Figure D.1). Hence, the decision tree will be constructed according to the specific aims of the study.

Therefore, in this thesis, different sentinel organisms and methods have been chosen (Figure D.1) in order to address the specific objectives of each Chapter.

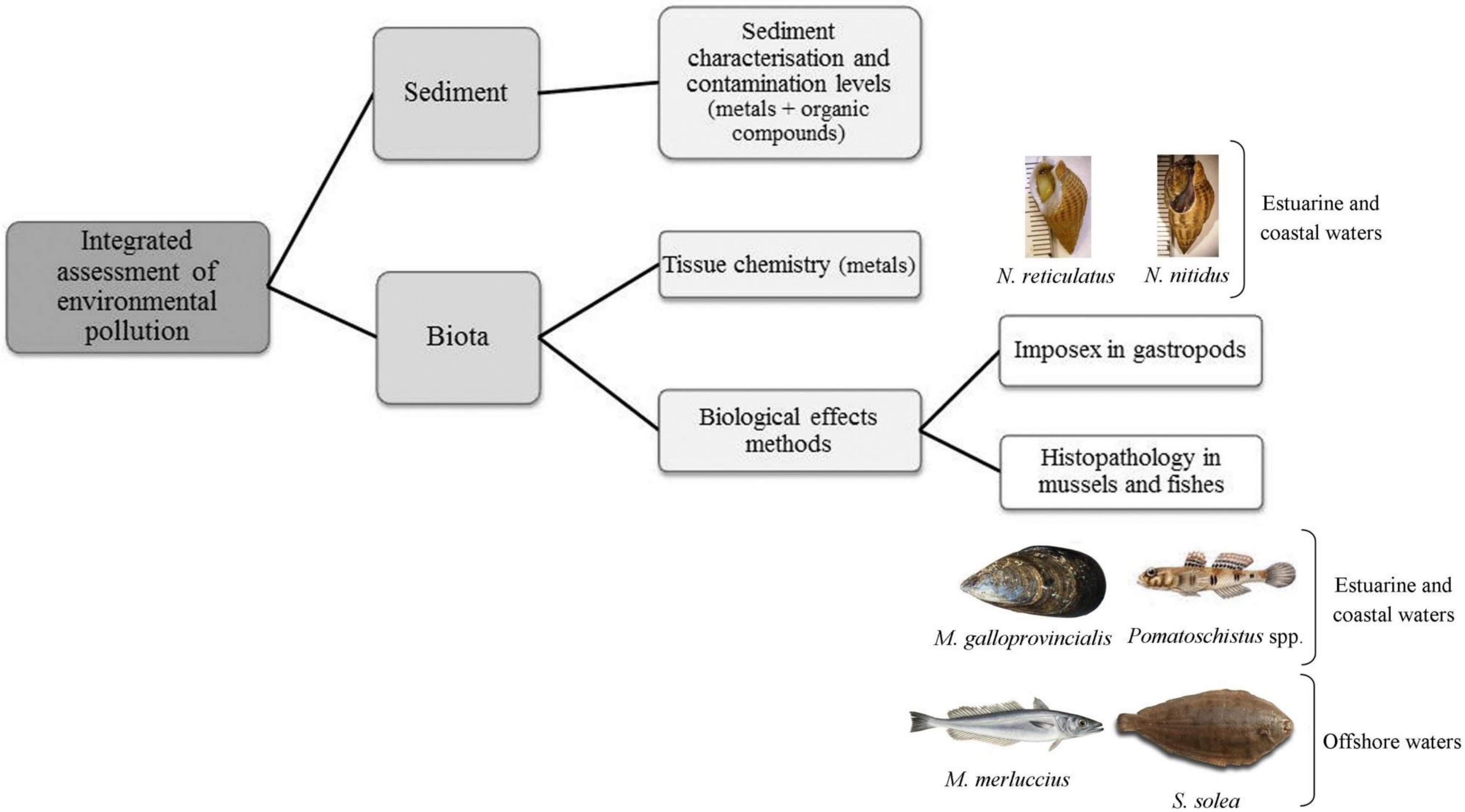

Figure D.1. Integrated assessment of the environmental pollution scheme proposed to cover estuarine, coastal and offshore areas of the Basque coast.

In an attempt to determine the effectiveness of the TBT ban (Regulation EC 782/2003) within the Basque coast, two gastropod species, *N. reticulatus* and *N. nitidus*, covering estuarine and coastal environments, were used in Chapter 1 as sentinel organisms to assess imposex levels and organotin tissue chemistry after the TBT prohibition (Figure D.1). The use of gastropods for the assessment of a specific biological effect, *i.e.* imposex levels, caused by organotin exposure within estuarine and coastal environments is very common (Barroso et al., 2005; Couceiro et al., 2009; Rodríguez et al., 2009a, 2009b; Ruiz et al., 2008; Sousa et al., 2009; Strand et al., 2006). Indeed, the assessment of imposex levels in gastropod species is regarded as a well-studied, accurate and cost-effective biological measurement (ICES, 2009), being thereby mandatory in the North Atlantic region (OSPAR, 2010). According to the outcomes of Chapter 1, although the imposex levels (measured during the same season as in 2007) were reduced in 2011 after the implementation of the European TBT ban within the study area, the BDI based on the organotin bioaccumulation results indicated the possibility of fresh TBT inputs. The sources of these recent TBT inputs may come from sediment desorption, dredging activities and/or illegal use of prohibited antifouling paints. Nonetheless, further research is required to determine the effectiveness of the European TBT regulation. In this respect, the determination of current organotin contamination levels of superficial sediments could provide valuable and potential information to better understand the temporal trends of organotin tissue residues and imposex levels in gastropods from the Basque estuaries and coast.

In order to assess the general health status of estuarine and coastal environments, mussels are used as sentinel organisms. Mussels are commonly employed in marine pollution monitoring programmes due to their ability to tolerate a wide range of environmental conditions and respond in a way that can be measured using biomarkers such as histopathological parameters (Brenner et al., 2014; Cappello et al., 2013; Garmendia et al., 2010; Múgica et al., 2015; Zorita et al., 2006). Therefore, in Chapter 2, *M. galloprovincialis* mussel was used to assess the general health status of Basque estuarine and coastal areas. As far as we know, there is no detailed histopathological semi-quantitative approach established for assessing the health status of mussels. In this attempt in Chapter 2, semi-quantitative histopathological indices were developed, adapted and validated in the digestive gland and gonad of mussels collected from five sites with different pollution levels. This histopathological approach allowed the integration of multiple histopathological traits into a single index, considering the biological importance and dissemination degree of the microanatomical features. Hence, these findings permitted a more sensitive and conclusive discrimination among sampling sites than the calculation of the prevalence of lesions and provided a health status ranking consistent with environmental parameters such as sediment properties, hydrodynamics and source of contamination. As expected, histopathological indices revealed that mussels from the most impacted sites, Getxo and Pasaia, endured the most significant deleterious effects. However, mussels from Gorliz, considered an area with minor disturbance, showed lower histopathological and metal bioaccumulation levels. These types of histopathological approach have already been developed in different fish species (Bernet et al., 1999; Costa et al., 2011; van Dyk et al., 2007) and, to a lesser extent, in invertebrates (Costa et al., 2013). On the other hand, within this Chapter, the confounding factors that may hamper this histopathological data were also established. As such, parasitosis and seasonal variations were identified as confounding factors and therefore, they should be carefully considered for biomonitoring the general health status of mussels within the Basque coast. Parasitosis, in particular, masked the real histopathological tissue damage of mussels; underestimating the histopathological indices (*i.e.* lower histopathological damage) in high polluted sites, whereas overestimating them (*i.e.* higher histopathological damage) in low impacted sites. On the other hand, seasonal variations demonstrated that inter-site differences were more pronounced in autumn when natural physiological responses of advanced maturation stages do not interfere in the histopathological response. Another important issue in assessing mussels' general health status is the choice of the target organ. In this study, the digestive gland presented more conclusive histopathological results than the gonad supporting the role of this organ in contaminant detoxification.

In order to assess the general health status of marine environments (*i.e.* estuaries, coast and offshore areas), fishes are appropriate sentinel organisms (Ferreira et al., 2004; Lang et al., 2006; Linde-Arias et al., 2008; Stentiford et al., 2014). Thus, a diverse range of fish species have been used as sentinel organisms in ecotoxicological studies, not only due to their species-specific geographical distribution, their ecological relevance or their commercial value in different regions, but also to their physiological adaptation to the extremely high variability in the physical environment and their ability to act as surrogates for higher vertebrates (Costa et al., 2009; Ferreira et al., 2004; Fonseca et al., 2014; ICES, 2006; Stentiford et al., 2014). However, mobility and migration of fishes may cause difficulty in pinpointing a pollutant as the cause of adverse effects in individuals or a population (Kirby et al., 2003). This shortcoming could be solved in some cases with the use of caging techniques (Beyer et al., 1996; Cazenave et al., 2014; Hylland et al., 2008). Within this thesis, three fish species were employed with the aim of assessing the general health status of different marine environments (Figure D.1): gobies (*Pomatoschistus* spp.) for estuarine and coastal systems (Chapter 3) and common sole (*S. solea)* and European hake (*M. merluccius)* for offshore waters (Chapter 4 and 5).

Gobies are emerging sentinel species that have been previously employed in a few toxicological research studies within estuarine areas due to their benthic and fairly territorial behaviour (Dolbeth et al., 2007; Fonseca et al., 2011; Martinho et al., 2006; Saaristo et al., 2010; Stentiford et al., 2003). Hence, in Chapter 3, the use of multi-organ histopathology in gobies (*Pomatoschistus* spp.) in combination with tissue and sediment chemistry was evaluated with the purpose of contributing to an integrated assessment of environmental pollution of the Ibaizabal estuary. As a result, Chapter 3 showed that sediments from the four areas of the Ibaizabal estuary were strongly and moderately impacted by metals and organic compounds, indicating that adverse biological effects to the resident biota could be likely. Accordingly, similar metal bioaccumulation levels and multi-organ histopathological indices were detected in gobies collected along the four transects of the estuary, indicating a similar affection degree. This is possibly due to the fact that gobies move and reflect the general health status of the whole Ibaizabal estuary since this study area is relatively short (22 km). However, histopathological indices and metal bioaccumulation levels were reduced in the more recent campaign (held in 2013) reflecting a lower impact on fish health status, which is consistent with the recovering status of the Ibaizabal estuary and the recent attempts to ameliorate this area (*e.g.* wastewater treatment and management). Liver, gills and kidney attained higher histopathological damage than spleen and gonad, highlighting their implication in pollutant detoxification processes or in their direct and constant contact with the surrounding environment. Fat vacuolation of hepatocytes, lamellar fusion and melanomacrophage centres were the most prevalent hepatic, branchial and renal alterations, respectively. These histopathological changes may indicate exposure to non-specific toxicants, although the influence of other unknown environmental factors should not be excluded. No pre- or neoplastic lesions were recorded in gobies collected in the Ibaizabal estuary, possibly due to the short life span of these organisms (Dolbeth et al., 2007) and the relatively slow formation of neoplastic lesions (Myers et al., 1987). Thus, non-neoplastic alterations have been proposed as useful and valuable histopathological biomarkers for health status assessment in gobies (Stentiford et al., 2003). Overall, the use of multi-organ histopathology in gobies, in combination with metal bioaccumulation and sediment contamination levels, contributed to a better understanding of sub-lethal effects and a more accurate environmental risk assessment in the Ibaizabal estuary. Nevertheless, further research is required for the determination of threshold values (baseline levels and EAC) and potential confounding factors of histopathological measurements to implement the use of gobies as sentinel species within Basque estuaries.

The assessment of the health status of marine offshore waters involves many logistical difficulties that may hinder and complicate the sampling strategies in these areas. Consequently, offshore regions are not frequently well assessed, as the evaluation of these areas poses a great challenge that should be overcome in order to respond to recent environmental policies such as MSFD. For this reason, in Chapter 4, a two-year campaign was carried out along the Basque continental shelf, in which biological effects methods (liver and gonad histopathology) in common sole (*S. solea*) and European hake (*M. merluccius*) together with tissue and sediment chemistry were determined for the integrated assessment of environmental pollution. Results indicated that although non-specific and early non-neoplastic toxicopathic lesions were observed in both species, common sole presented higher metal bioaccumulation and histopathological lesions in the liver than European hake, indicating a higher sensitivity to stressor exposure. However, both fish species were suitable and complementary candidates as sentinel organisms for biomonitoring biological effects of marine sediments and deep water column within the Basque continental shelf (Chapter 4). On the other hand, European hake is an easy to catch fish species due to its abundance within the Basque continental shelf, which may reduce sampling logistical difficulties, although owing to its migratory condition it may not reflect the biological effects of a specific-site. Common sole, however, demonstrates habitat fidelity and sensitivity, which is beneficial for biomonitoring purposes, and therefore it may be considered as a more suitable fish sentinel species than European hake.

Within this Chapter the utility of two existing histopathological indices (Costa et al., 2009 and Lang et al., 2006) was also evaluated since methodological comparisons or intercalibration exercises are necessary to attain quality assurance of biological effect measurements in biomonitoring approaches (Mathiessen, 2000). Likewise, both histopathological indices were accurate, conclusive and reliable tools for reflecting the health status of the Basque offshore environments. Both histopathological indices used a similar severity classification system consisting of three categories (slight, moderate and severe) of microanatomical alterations, which contributes to the significant correlation between them. Thus, the two existing histopathological indices may be indifferently used. The coherence between both approaches may be due to the fact that the most challenging step in histopathological studies is the establishment of correct diagnosis criteria, which requires special training and participation in intercalibration exercises.

As for the integrated assessment of environmental pollution, the Basque continental shelf could be considered a minor disturbed area. Sediments were found to be moderately impacted by metals, but non-impacted by organic compounds. Furthermore, the lack of correlation between sediment contamination levels, bioaccumulation and histopathological indices suggested that other factors, rather than pollution alone, were responsible for the biological effects observed. Furthermore, the dilution and dispersion capacities of Basque offshore environments (Valencia et al., 2004) together with a low impact of human pressures (Borja et al., 2006, 2011; Legorburu et al., 2013) reinforce this idea. Even so, as stated in Chapter 3, the establishment of threshold values and confounding factors are needed for correct interpretations.

In this regard, Chapter 5 provided the baseline levels of histopathological traits in the liver and gonad, together with biometric parameters and metal hepatic bioaccumulation levels of common soles collected over a one-year period along the Basque continental shelf. According to the outcomes, biometric parameters, biological indices (HSI and GSI) and the gonad histopathological index varied according to reproductive cycle and gender. In agreement, Zn, Cd, Cu, and Pb demonstrated a seasonal bioaccumulation pattern in both genders, which mandates caution when interpreting these findings without considering natural biological and environmental variations. In turn, season, gender and age did not act as confounding factors in the liver histopathological index which is beneficial for biomonitoring purposes. This could be particularly useful for the evaluation of impacts on biota physically affected by acute pollution events such as oil spills. As for organ comparison, the liver endured more severe histopathological damage than the gonad. However, most of the alterations were classified as non-specific and early non-neoplastic toxicopathic lesions, sustaining the notion that the Basque continental shelf may be regarded as a reference area. Nevertheless, in the present study one case of adenoma and one case of intersex were recorded for the first time in common sole which indicates the susceptibility of this species to develop toxicopathic pathologies. The incidence of adenoma and intersex in flatfish species is strongly associated with the presence of environmental pollutants (Allen et al., 1999; Johnson et al., 2008; Koehler et al., 2004; Stentiford and Feist, 2005), although these alterations have been also observed in presumably pristine waters (Schwindt et al., 2009). Therefore, further studies with epidemiologically significant number of fish individuals, historical data sets and concomitant analysis are required to investigate relationships between contaminant burdens and lesion occurrence.

As the main contribution of this thesis is to the development of histopathological indices in different tissues and organisms, this issue deserves a deeper explanation. As such, it must be noted that the histopathological indices used in Chapters 2 to 5 are mainly adapted from the indices developed by Costa et al. (2009). This histopathological approach is cumulative and accounts not only for the number of alterations observed in each individual, but also for their relative importance. Only persistent or frequent alterations within an organ are scored, discarding those that appear occasionally or sporadically. Therefore, the selection of suitable histopathological features is of great relevance for the estimation of histopathological indices in order to achieve comparable, reliable and conclusive findings. This latter was especially important in the standardised histopathological index used in mussels (Chapter 2), since the cumulative histopathological damage was divided by the maximum value, which may change if new histopathological features are included. Therefore, the same histopathological lesions ought to be considered for comparison purposes. However, standardised and non-standardised histopathological indices are appropriate biological and effective tools for biomonitoring scopes, although owing to its easier estimation procedure, the non- standardised histopathological index is mainly used within this thesis (Chapters 3, 4 and 5).

On the other hand, as we have already stated within this study, the determination of baseline levels and potential confounding factors of biological effect measurements is a crucial issue within biomonitoring approaches. Therefore, the findings of Chapter 1 were compared with the baseline values previously determined by OSPAR for the assessment of TBT effects in gastropod species, as well as with the classification scheme or boundaries (EAC) based on the VDSI and TBT levels (OSPAR, 2004). As the rest of the Chapters of this thesis have not defined threshold levels for the histopathological assessment in sentinel species (*i.e.* mussels, gobies, European hake and common sole), this thesis made a step forward in this matter. For mussels (Chapter 2) and common soles (Chapter 5), the baseline levels and confounding factors were established by means of seasonal studies. For gobies (Chapter 3) and European hake (Chapter 4), however, the interpretation of histopathological effects is a more difficult task, since there is a major gap in knowledge concerning the biology of these species. This downside or uncertainty may be solved with (*1*) a suitable sampling design (*i.e.* sampling fish from similar size during the same season) to reduce the potential confounding factors; and (*2*) with the integration of different LOEs which combine different information to complete the different pieces of the jigsaw. However, further research is required for the determination of baseline levels and potential confounding factors of biological measurements to implement the use of gobies and European hake within marine biomonitoring approaches. In addition, a further challenge to establish classification schemes (EAC) for histopathological assessment in sentinel species, except in gastropods, is evidenced within this thesis for the purpose of translating this complicated biological data into a more interpretative and receptive message for stakeholders and policy-makers.

Overall, the tiered approach proposed in this thesis may be useful in deciding how to design the integrated assessment of environmental pollution in the Basque coast. In particular, the assessment of imposex levels and organotin tissue chemistry in gastropods and histopathology in mussels and fishes in combination with tissue and sediment chemistry reflected solid assessment of TBT exposure or global health status of the Basque marine environments in an integrative way. On the other hand, baseline levels and the influence of several confounding factors in sentinel organisms was determined for a correct data interpretation, but the need to establish and apply EAC for the identification of pollution-induced responses or other potential confounding factors is evidenced. Thus, the findings could contribute and help to monitor compliance with the premises of environmental policies such as Descriptor 8 in the MSFD. However, a further effort to integrate biological effects of pollutants (or Descriptor 8) with the rest of MSFD Descriptors through an ecosystem-based approach should be made in order to achieve GES of marine environments, and attain useful information for stakeholders and policy-makers for effective management of the ecosystem in this region (Borja et al., 2011; Ferreira et al., 2011).

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Further recommendations

Taking into account the outcomes of this Thesis, in order to achieve a more cost-effective, accurate and reliable assessment for upcoming biomonitoring approaches within the Basque marine environment, some recommendations are provided below and summarised in Table F.1.

For the evaluation of the effectiveness of TBT prohibition, the determination of imposex levels in gastropods is recommended since this measurement is cheaper and faster than chemical analysis. However, in cases in which imposex levels are exceeded, chemical analysis may also be carried out in sediment or biota in order to elucidate the reasons for exceedance of this antifouling paint. The use of either gastropod species is suitable as sentinel organisms, but *N. nitidus* is more appropriate for inner estuaries with low salinity ranges, whereas *N. reticulatus*, due to its marine character, is more suitable for outer estuaries and coast.

Nevertheless, the number of sampling sites assessed within this thesis should be reduced and, only locations with signs of TBT exposure, such as harbours with high maritime traffic, may be surveyed (*i.e.* Zierbena, Bilbao, Getaria, Donostia and Pasaia). The best sampling period for imposex determination is when gastropods show gametes at spawning stages, from February to June (Table F.1.). During this period their sexual characters are easily distinguished, which facilitates gender differentiation. Additionally, as these studies have been carried out during these months, during the first half of the year, the influence of seasonality is reduced in inter-annual comparisons.

On the other hand, if adult individuals (> 4 years) are employed for imposex assessment, considering the fact that imposex is practically irreversible, there is no need to repeat the surveys very frequently (> 4 years), since the same age cohorts will be resampled and unreliable temporal trends of TBT exposure will be evidenced. Finally, the imposex levels obtained should always be compared with the threshold levels (baseline and EAC).

In order to assess the general health status of estuarine, coastal and offshore environments, the use of histopathology in diverse sentinel organisms (*i.e.* mussels and fishes) is recommended. Mussels being sessile filter feeders are used as indicators of contamination levels of estuarine and coastal environments (Table F.1.). Furthermore, the measurement of organic and inorganic compounds may give clues to identify the causative agent of the histopathological alterations. As for selecting the target organ, digestive gland is more sensitive than gonad, although the latter should also be analysed for intersex condition and gamete developmental assessment. The same sampling locations as those surveyed in Chapter 2 should be maintained to assure the coverage of the Basque coast. Besides, the most suitable sampling period to avoid seasonal effects related with the reproductive cycle is autumn, when mussels show gametes at resting stages. An annual sampling frequency is believed to be appropriate to assess the impacts of different anthropogenic activities. However, for a correct histopathological assessment, EAC should be established.

Additionally, gobies are promising sentinel fish species for the assessment of environmental pollution within Basque estuarine environments. They are very abundant and due to their small size, gobies are directly kept in fixative, which facilitates the sample processing in field cruises. In contrast, the subsequent histological processing is more laborious, especially when the spine is cut (Table F.1.). Thus, sample decalcification trials should be conducted to improve the quality of the sections. Species identification is critical too, but genetic analysis could be carried out in parallel using a small piece of the tail to determine whether different gobies species present different

Table F.1. Schematic guidance for future integrated assessments of environmental pollution within the Basque marine environments.

Method	Imposex	Imposex	Histopathology	Histopathology	Histopathology	Histopathology
Indicator	TBT exposure	TBT exposure	General health status	General health status	General health status	General health status
Sentinel organisms	*N. nitidus*	*N. reticulatus*	*M. galloprovincialis*	*Pomatoschistus spp.*	*M. merluccius*	*S. solea*
Study area	Inner estuaries	Outer estuaries and coast	Estuaries and coast	Estuaries and coast	Offshore waters	Offshore waters
Target organ	Gonad	Gonad	Digestive gland and gonad	Liver, gills, kidney and gonad	Liver and gonad	Liver and gonad
Survey frequency	Every 4 years	Every 4 years	Annually	Annually or biannually	Annually	Annually
Sampling period	Winter and spring	Winter and spring	Autumn	Autumn	Summer	Summer
Number of sampling locations/areas	Reduce the survey to the sampling sites in which imposex and TBT levels were exceeded (11 sites)	Reduce the survey to the sampling sites in which imposex and TBT levels were exceeded (3sites)	The same 5 locations surveyed in the Basque coast (Getxo, Gorliz, Mundaka, Pasaia, Hondarribia)	Two transects in the Ibaizabal estuary	One location in the Basque continental shelf	One location in the Basque continental shelf
Confounding factors	Season	Season	Season and parasitosis	Non-determined	Non-determined	Season and gender
Baseline levels	Determined by OSPAR	Determined by OSPAR	Determined in this thesis	Non-determined	Non-determined	Determined in this thesis
EAC	Determined by OSPAR	Determined by OSPAR	Further research	Further research	Further research	Further research
Complementary chemical data	TBT, DBT and MBT in biota and sediment	TBT, DBT and MBT in biota and sediment	Metals, PAHs and PCBs in biota and/or sediment	Metals, PAHs, PCBs and organochlorides in biota and/or sediment	Metals PAHs, PCBs, organochlorides and bile metabolites in biota and/or sediment	Metals PAHs, PCBs, organochlorides and bile metabolites in biota and/or sediment
Limitations	Imposex assessment requires expertise	Imposex assessment requires expertise	Expertise is required Difficulties in species identification	Difficulties in histological processing	Migratory species	Difficulties in identification small specimens
Advantages	Imposex is rapid and cost-effective	Imposex is rapid and cost-effective	Mussels are easy to sample	Abundant benthic species Sedentary	Abundant commercial species	Abundant benthic species in estuaries and coast

sensitivities. Due to the short life cycle of gobies (< 2 years), only non-neoplasic alterations may be considered within histopathological indices estimation (Table F.1.). As for target organ selection, liver, gills and kidney are more responsive to pollutants than spleen or gonad. However, for chemical analysis a pool of entire individuals is required. On the other hand, due to the short length of Basque estuaries and the short seasonal migrations of gobies, the number of sampling transects within the Ibaizabal estuary should be reduced, from 4 transects to 2. The most suitable period to carry out the survey is autumn, when gobies are in early gametogenesis and the influence of the reproductive cycle is minimised. Nevertheless, in order to implement the use of gobies for the assessment of estuarine environmental pollution, further research is needed to establish the influence of confounding factors and threshold values.

For the evaluation of the health status of offshore waters, the use of European hake and common sole could be complementary. However, the common sole due to its benthic behaviour presents a higher sensitivity to pollution than the European hake, and thus, it is highly recommended. Although the European hake is a very abundant commercial species, due to its long migrations the causal-effect relationship between a contaminant and biological effects is not clear. In contrast, the common sole is more sedentary and histopathological alterations induced by contaminants are more severe in the liver than in the gonad, which reveals the implication of this organ in pollutant detoxification. In combination with histopathology, chemical analysis should be performed in the liver for metal and PCB determination, while bile could be used for the measurement of PAHs.

For future biomonitorings, a unique sampling area, rather than two sites would be more cost-effective. Indeed, no significant differences were recorded in the histopathological alterations or hepatic metal bioaccumulation levels of common soles or in the contaminant levels determined in sediments from the west and east of the Basque continental shelf. As for the sampling period, the most suitable season is summer when common soles show gametes in early gametogenic stages. But still, further research is needed to establish threshold values to differentiate pollution induced effects from natural environmental variations.

Conclusions and Thesis

Taking into account the objectives of this thesis and the questions that it aimed to answer, we can conclude that:

1. The imposex levels in *N. reticulatus* and *N. nitidus* were reduced after the implementation of the European TBT ban (EC 782/2003) within the Basque coast. Still, the BDI results based on the organotin tissue chemistry indicated the possibility of fresh TBT inputs. These recent TBT inputs may be due to sediment desorption, dredging activities or illegal use of prohibited antifouling paints. However, further research is needed to determine the effectiveness of the European TBT regulation.

2. The integration of multiple histopathological traits into single indices provided a sensitive discrimination of the health status of mussels (*M. galloprovincialis*) from different sampling sites of the Basque coast consistent with environmental variables (*e.g.* sediment characteristics, hydrodynamics and source of contamination).

3. Parasitosis and seasonal variations act as confounding factors in the histopathological assessment and thus, they should be carefully considered for evaluating the health status of mussels within the Basque estuaries and coast. Hence, the findings revealed that parasitosis masked the real tissue damage, underestimating the histopathological indices in highly polluted sites (*i.e.* slighter tissue damage) and overestimating (*i.e.* more severe tissue damage) them in slightly impacted sites. On the other hand, inter-site differences were more pronounced in autumn, when natural physiological responses of advanced maturation stages did not interfere with the histological response.

4. The use of the multi-organ histopathology in gobies (*Pomatoschistus* spp.) in combination with metal bioaccumulation and sediment chemistry, contributed to the understanding of sub-lethal effects and a reliable environmental risk assessment in the

Ibaizabal estuary. Gobies collected along the Ibaizabal estuary were similarly impacted, but a lower affection degree was observed in the last campaign, which was in accordance with the recovery and recent attempts to ameliorate the area.

5. Liver, gills and kidney attained higher histopathological damage than spleen and gonad in gobies collected along the Ibaizabal estuary, highlighting their implication in pollutant detoxification processes and their suitability as target organs within biomonitoring approaches. Fat vacuolation of hepatocytes, lamellar fusion and melanomacrophage centres were the most prevalent hepatic, branchial and renal alterations. These alterations indicate exposure to unspecific pollutants, although the influence of other unknown environmental factors should not be excluded.

6. Common sole (*S. solea*) and European hake (*M. merluccius*), two ecological and commercial target species, were good and complementary candidates as sentinel organisms for biomonitoring biological effects of marine sediments and bottom water column areas within the Basque continental shelf. However, common sole manifested higher sensitivity to low environmental pollution than European hake, possibly due to its benthic behaviour. Nevertheless, several drawbacks such as sampling logistical difficulties, mobility of sentinel organism and non cost-effective conditions may hinder and complicate data collection and interpretation.

7. The use of semi-quantitative histopathological indices integrating all lesions reflected the health status of common sole and European hake of the Basque continental shelf. The comparison of two existing histopathological indices (Costa et al., 2009 *versus* Lang et al., 2006) ensured data quality assurance, since both approaches were accurate and reliable indicating that both histopathological indices may be applied interchangeably. This is probably due to the fact that the most critical issue in attaining comparable and good quality data in histopathological assessments is diagnosis, which requires expertise and special training.

8. Fish histopathology, together with sediment and tissue contamination levels, was a suitable approach to assessing environmental pollution of Basque marine environments in an integrative way. The lack of correlation between sediment contamination levels, metal bioaccumulation and histopathological indices in common sole and European hake supported the notion that the Basque continental shelf may be regarded as a pristine environment.

9. Prevalence of liver and gonad histopathological traits, gonad histopathological index, biometric parameters and hepatic metal bioaccumulation levels were modulated by season and gender in common soles collected along the Basque continental shelf, which indicates that sampling period and gender differentiation should be thoroughly considered in sampling design and subsequent data interpretation. The most suitable period for sampling is likely during the early-mid gametogenesis, from June to September. Conversely, season, gender and age did not interfere in the liver histopathological index assessment, which is beneficial for biomonitoring approaches, especially for the evaluation of pollution events such as oil spills.

10. The determination of baseline histopathological levels using common soles as sentinel organisms provided reference values required to differentiate pollution-induced effects from natural responses. However, for future biomonitoring, the need to establish EAC is highlighted.

Thesis

"Integrated assessment of environmental pollution using biological effect methods (*i.e.*imposex or histopathology), in combination with tissue and sediment chemistry, provides holistic and reliable evaluation of the TBT exposure in gastropods or general health status in mussels and fishes within the Basque marine environments. Nevertheless, in order to include this approach within European Directives (WFD and MSFD) the need to establish the influence of confounding factors, as well as threshold values, is highlighted"