UNIVERSIDAD DE MURCIA

FACULTAD DE BIOLOGÍA

Effect of confounding factors in biological responses of mussels, *Mytilus galloprovincialis*, to pollution: Implication in large-scale monitoring programs

Factores de confusión en la respuesta biológica del mejillón, *Mytilus galloprovincialis*, a la contaminación: Optimización de los protocolos de monitorización biológica

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Ever try,

Ever failed.

No matter.

TRY AGAIN!

Fail again,

Fail better.

Samuel Beckett

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SUMMARY

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SUMMARY

The main objective of this thesis was to identify how ***mussel condition*** is affecting biomarker responses to pollution. For this purpose, some specific objectives were proposed:

1. To assess the main biological variables that could be acting as confounding factors in monitoring programs by establishing relationships between i) biological variables and biomarkers, and ii) biomarkers and pollution, using wild mussel populations.

2. To study the effect that ***mussel nutritive state (condition I: quantity)*** has on biomarker responses to pollution under controlled laboratory conditions.

3. To study the effect that ***mussel nutritive state (condition II: quality)*** has on biomarker responses to pollution under controlled laboratory conditions.

4. To assess the effect of the ***mussel reproductive state (condition III)*** on biomarker responses to pollution under controlled laboratory conditions.

In order to cover these objectives, a battery of biomarkers at different levels of biological organization was considered for a better interpretation of pollution effects on mussels: biochemical, immunological, physiological and whole organism responses.

Results obtained in this thesis evidenced for the first time a *great geographical variability* in the biology of wild mussel populations used to assess pollution in large-scale monitoring programs. The larger the area sampled by a monitoring program, the higher the variability of biological parameters observed, which is related to the natural variability of environmental factors, mainly food availability. The strong relationship observed between biomarker responses and mussel biology evidenced the presence of *confounding factors* modifying the expected biomarker responses to pollution.

In addition, laboratory experiments were designed to obtain more information about the effect that mussel condition has on biomarker responses. Results showed that ***mussel condition***, in terms of food quality, did not affect the *immune biomarkers.* However, mussel nutritive state considering both, food quality and quantity, strongly affected physiological and biochemical biomarkers. The biomarker *SFG* was highly dependent on mussel nutritive state. This fact, compromises the SFG use in large-scale monitoring programs, which are characterized by a great variability in trophic conditions between sites and between sampling periods. In the same way, biochemical biomarkers were strongly dependent on food quality and quantity, specifically *CAT, SOD, GPx* and *GR*. On another hand, mussel reproductive state did not affect physiological biomarkers. Conversely, a strong effect of reproduction was observed in biochemical biomarkers showing higher values at reproductive stage.

Bioaccumulation was also dependent on the environmental characteristics of the reproduced scenarios. The pollutant showed higher accumulation in mussel with higher condition and at resting stages. As a consequence, pollutants are not only accumulated in biota as a function of their environmental concentrations, but also depending on mussel nutrition and reproduction.

In general, the effect of mussel condition was higher than the effect of pollutant exposure in most of the studied biomarkers, mainly physiological and biochemical biomarkers. Moreover, the effects of toxicant were different depending on mussel condition (nutritive and reproductive state). This explains the difficulty observed in field studies to establish relationships between biomarkers responses and pollution. Thus, it is necessary to consider ***mussel condition*** as an important variable to take into account in monitoring programs, mainly in large-scale monitoring programs where different mussel conditions are coexisting at the same time, for the proper interpretation of biomarker responses to pollution.

INTRODUCTION

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INTRODUCTION

1. What is the problem?

1.1. Marine pollution

The coast, as the interface between land and sea, is recognized as one of the most important ecosystems on the Earth, providing a wide range of services for humans. In the United Nations Convention on the Law of the Sea (Montego Bay, 1982) it was defined ***pollution of the marine environment*** as "*the introduction by man, directly or indirectly, of substances or energy into the marine environment, including estuaries, which results or is likely to result in such deleterious effects as harm to living resources and marine life, hazards to human health, hindrance to marine activities, including fishing and other legitimate uses of the sea, impairment of quality for use of sea water and reduction of amenities"(article 1.4).*

1.2. Major classes of pollutants

Many different chemicals are regarded as pollutants, ranging from simple inorganic ions to complex organic molecules. In this context, it is possible to distinguish some important groups: inorganic ions, metals, organobromates, organochlorinates, hydrocarbons, current use pesticides, detergents and pollutants of emergent concern.

1.2.1. Metals and inorganic ions

The main inorganic ions are chlorides, sulphides, nitrates, phosphates and carbonates. Within this group, some acid and basic waste can be also included, as well as toxic gases such as ammonia, nitrogen or chlorine, among others. They are not particularly toxic but may cause environmental problems because they are used in large quantities. These ions come from domestic and industrial discharges, as well as from soil erosion (Walker *et al*, 2000).

Metals are defined by chemists as elements which are good conductors of electricity and generally enter chemical reactions as positive ions or cations (Clark, 2001). Within this category, it is possible to differentiate between ***essential metals*** and ***non-essential metals*** (Ansari *et al*., 2004). The essential metals are present in small quantities in organisms as micronutrients such as Cd, B, Co, Cr, Cu, Mo, Mn, Ni, Se and Zn which have biological function in the organisms. On the other hand, non-essential metals (e.g: Hg, Pb, Sb and Bi) do not have recognized biological function.

As consequence of their potential harmful effect, some of these elements (Hg, Cd, Pb, Cu, Zn and As…) have been considered of prioritary concern at international level. For example, they have been included in the priority substances list of the Water Framework Directive (WFD) and other international agreements for the protection of the marine environment such as the OSPAR and Barcelona Conventions. In general, the

presence of some (Cd, Cu, Zn and As) of these metals in the marine environment has an important natural input, coming from rocky or mineral deposits. Although metals occur naturally in the environment, the use of metals by human, particularly since the industrial age, has resulted in excessive releases. Anthropogenic sources of metals include fossil fuel and waste burning, mining and ore processing, chemical production, and agriculture (Kimbrough *et al*., 2008). Specially, some of them (Hg and Pb) received higher attention due to its toxic effect on organisms. Natural inputs of Hg in the oceans come from mercury-bearing rock and degassing of the Earth crust, in detail through volcanic activity. Moreover, it also comes from anthropogenic sources mainly mining activity and industrial and metallurgical processes. However, since the discovery of the risk of Hg to human health, since the 50s, there has been a reduction of human inputs controling and eliminating sources of Hg (Clark, 2001). Similarly, inputs of Pb come from natural (mineral deposits) and anthropogenic (minery and widely used in industrial processes as well as the manufacture process of gasoline), but the main source of Pb are due to the gas emission from gasoline combustion (Mao *et al*., 2009) and smelting processes which include Pb (USEPA, 2003). Nowadays the use of Pb in gasoline is restricted, following the guidelines established by international agencies such as the ***United Nations Conference on Sustainable Development*** (ONU, 1994).

1.2.2. Organobrominated compounds

Brominated flame retardants (BFRs) are a heterogeneous group of chemical substances which are used in industrial processes to retard the combustion and reduce the flammability, as well as other insulation products (De Wit, 2002). There are different types of retardants but, among the most used ones, polybrominated diphenyl ethers (PBDEs) have received special attention (Clark, 2001). There are around 209 PBDEs congeners, but only some of them are used in industrial processes. As a consequence of its high persistence in the environment, some representative congeners (BDE28, BDE47, BDE66, BDE85, BDE99, BDE100, BDE153, BDE154 and DBE183) have been considered to assess the quality of the marine environment (JAMP, MEDPOL) and their concentration is expressed as the sum of the nine congeners: Σ_9PBDE. This type of compounds is released to the atmosphere during the production processes and also during incineration processes. In addition, they can also come, in lower concentrations, from landfills through runoff and infiltration processes (Alaee *et al*., 2003).

1.2.3. Organochlorinated compounds

The organochlorinated compounds are a group of persistent organic compounds in which are included the polychlorinated biphenyls (PCBs), the organochlorinated pesticides (such as hexachlorocyclohexanes –HCHs-) or the organochlorinated insecticides (such as dichloro dyphenyl trichloroethane and its metabolites –DDTs-). In general, the main characteristic of this group is that they are synthetic compounds extensively used in industry and agriculture. Its main characteristic is their toxicity and

persistence in the environment. They have a reduced solubility and high lipophilicity, being harmful for organisms (Clark, 2001).
PCBs are compounds derived exclusively from anthropogenic sources that consist of 1 molecule of biphenyl to which between 1 and 10 atoms of chlorine are attached. They were extensively produced since 1930 for their industrial applications as heat transfer fluids. However, since the 80s, they have been banned by the European Union (WHO, 1993). This group of contaminant present several congeners, but only some of them have been considered to assess the environmental quality (PCB28, PCB52, PCB101,PCB118, PCB138, PCB153 and PCB180) as recommended by ICES (seven ICES indicator PCBs congeners), and are currently expressed as Σ_7PCBs.

The DDT was widely used as insecticide but was forbidden by the United States Environmental Protection Agency (US EPA) in 1972, and later also by Europe, with the discovery of its persistence in the environment and with the growing evidence of its ability to cause harmful side effects on living organisms (Walker *et al*., 2009). The DDT and its degradation products are considered in pollution monitoring programs: p′-DDE, p,p′-DDD, p,p′-DDT and p′-DDT.

Moreover, this group includes other pollutants considered toxic, which need to be regularly monitored, such as chlordanes (cis- and trans- chlordanes) and hexachlorocyclohexanes (HCHs: α-HCH and γ-HCH) (González-Quijano and Fumega, 1996).

The organochlorinates group includes some of the 12 prioritary persistent organic compounds (POPs) regulated by the Stockholm Convention (Stockholm Convention, 2001), compounds produced both intentionally and non-intentionally, whose production need to be reduced or its use banned. This constitutes the first evaluation from the chemical, toxicological and economic point of view, performed to restrict the production and use of POPs. The first list included: pesticides (aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, hexachlorobenzene, mirex, toxaphene), industrial chemicals (hexachlorobenzene, PCBs) and other products (polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans -PCDD/PCDF-). However, in 2009 the list was extended to 21 compounds distributed in 3 annexes (A: elimination, B: restriction and C: Unintentional production) in which also the α-HCH and γ-HCH were included as prioritary substances.

1.2.4. Hydrocarbons

Hydrocarbons are compounds that contain only carbon and hydrogen. They can be classified into two main groups: ***aromatic hydrocarbons*** that contain ring systems with delocalized electrons and ***non-aromatic hydrocarbons*** that do not contain a ring system (Walker *et al*., 2000). The polycyclic aromatic hydrocarbons (PAHs) are among the most important pollutants from this group. These compounds are formed by two or more fused aromatic (benzene) rings. They can come from natural sources but their

presence in the marine environment is mainly due to anthropogenic activity (Kennish, 1992). The largest releases of PAHs are due to the incomplete combustion of organic compounds during the course of industrial processes and other human activities. Important sources include the combustion of coal, crude oil, and natural gas for both industrial and domestic purposes. The release of crude oil into the sea by the offshore oil industry and the wreckage of oil tankers are important sources of PAH in certain areas. Other sources of PAHs pollution are forest fires, which may or may not be consequence of human activity but are a significant source of PAHs on the marine environment (Walker, 2009).

This group of contaminants present several congeners, but only some of them have been considered as priority pollutants to represent environmental quality: Σ_{12}PAHs (phenanthrene, anthracene, fluoranthene, pyrene, chrysene, benz [a] anthracene, benzo [b] fluoranthene, benzo [k] fluoranthene, benzo [a] pyrene, indeno [1,2,3-cd] pyrene, dibenz [ah] anthraceneandbenzo [ghi] perylene).

1.2.5. Current use pesticides (CUPs)

The regulation of previous substances used as pesticides, such as DDTs, promoted the development of alternative, less persistent, compounds (Hoferkamp *et al.*, 2010). This new group of substances was denominated ***current use pesticides*** (CUPs). Because of its high solubility and lower persistence in the environment they have replaced the use of previous, more persistent, compounds. Among the currently used pesticides, the organophosphorus insecticides (OPs) are the most widely studied (USEPA, 2011). OPs are used in agriculture, homes, gardens and veterinary practices. However, in the past decade, several notable OPs have been discontinued for use, including parathion and chlorpyrifos, which are no longer registered for agriculture and home use, respectively (Roberts and Aaron, 2007).

1.2.6. Detergents

Detergents are organic compounds extensively used in both domestic and industrial processes. The main entry of detergents to the sea is through sewage discharges. They contain surfactants or surface-active agents which are chemicals that reduce the surface tension of oil and water. Surfactants in detergents are toxic to aquatic life, persist in the environment and break down into additional toxic byproducts, according to US EPA (2014).

1.2.7. Pollutants of emergent concern

This group is composed by chemicals that, despite not having high persistence in the environment, are produced in a massive way, entering continuously into the sea (Barceló, 2003). Within this group, it is possible to find chemicals from both domestic and industrial sources, such as pharmaceuticals, plastic debris and radioactive

substances. In this context we may highlight the impact of pharmaceuticals due to its massive production. In fact, in Spain, the pharmaceutical sector is the industrial sector with higher research investment and development (Farmaindustria, 2010). After consumption, a fraction of the pharmaceuticals ingested by humans is excreted in its original form (Kumar *et al*., 2008) and, despite water is treated in waste water treatment plants (WWTP), there is not an effective treatment for the elimination of these compounds (Carballa *et al*., 2004), and thereby pharmaceutical waste enters the marine environment directly through sewage discharges.

Other important source of pollution of emergent concern is plastic litter. Some authors consider that we currently live in the *Plastic Age* (Cózar *et al*., 2014). Nowadays, plastic is the most abundant material found as marine debris (up to 80%), with an estimation of 4.8 to 12.7 million metric tons of plastic waste already present in the oceans, and a further 3-fold increase predicted by 2025 (Jambeck, *et al*., 2015). The accumulation of microplastics (MPs, <5 mm diameter) in the ocean is a consequence of either the direct release of small plastic particles (e.g. microbeads from cosmetics) or the fragmentation of larger items. At some locations, MPs are already reported at concentrations up 10^6 items km^{-2}, and their abundance is likely to increase (Law and Thompson, 2014). MPs are of environmental concern because their size renders them accessible to a wide range of organisms, and have potential for physical and toxicological harm (Law and Thompson, 2014).

In a similar way, there is an increasing concern about radioactive isotopes. Radioactivity is a natural phenomenon in the sea due to the activity of cosmic rays (Clark, 2001). However, since the development of nuclear energy and atomic weapons, the debate in relation to the safety of low levels of radioactivity in the environment has been increasing (Walker *et al*., 2000). Although it is a subject of concern, there are few studies regarding the ecological impact of radioactivity in the sea (Clark, 2001).

2. Inputs, pathways and fate of pollutants in the sea

The marine environment is considered as the ultimate receptor for pollutants. Pollution has been detected even in remote areas, such as the polar regions Stegeman *et al*., 2001).

Figure 1 shows a diagram with the main pathways, inputs and the distribution of pollutants into the marine environment. The most important pathways of pollutants into the sea are: direct outfalls from urban, industrial and port activities, rivers (pesticides and fertilizers from agriculture and forestry that are washed off the land or petroleum and oils from surface run-off), and the atmosphere, since pollutant emissions to the atmosphere are returned to the sea in form of rain, and gaseous wastes can be directly dissolved in the sea's surface microlayer (Walker *et al*., 2009).

After entering into the sea, and according to their characteristics, pollutants are normally accumulated within the marine environmental compartments (matrices): surface microlayer, water column, sediment and biota (Figure 1). Chemical molecules can suffer biological and/or physico-chemical changes that will influence their distribution in the environment. In this sense, ***bioavailablility*** can be defined as the fraction of a chemical compound present in the environment which can be incorporated by an organism (Walker *et al*., 2009). The bioavailability and spatial distribution of contaminants in the marine environment are therefore determined by hydrodynamics, biogeochemical processes and environmental conditions (Dachs and Mejanelle, 2010). For example, different inorganic (metals) and organic pollutants such as PAHs, PCBs, or pesticides, tend to join organic and inorganic particulate matter (Jensen, 2012). As a consequence, aquatic organisms are exposed to chemical pollutants by two different ways: dissolved pollutants in the water column can be taken up through the gills and body surfaces, and pollutants adsorved to organic or inorganic particles can be ingested and assimilated in the digestive tract (Van der Oost *et al*., 2003).

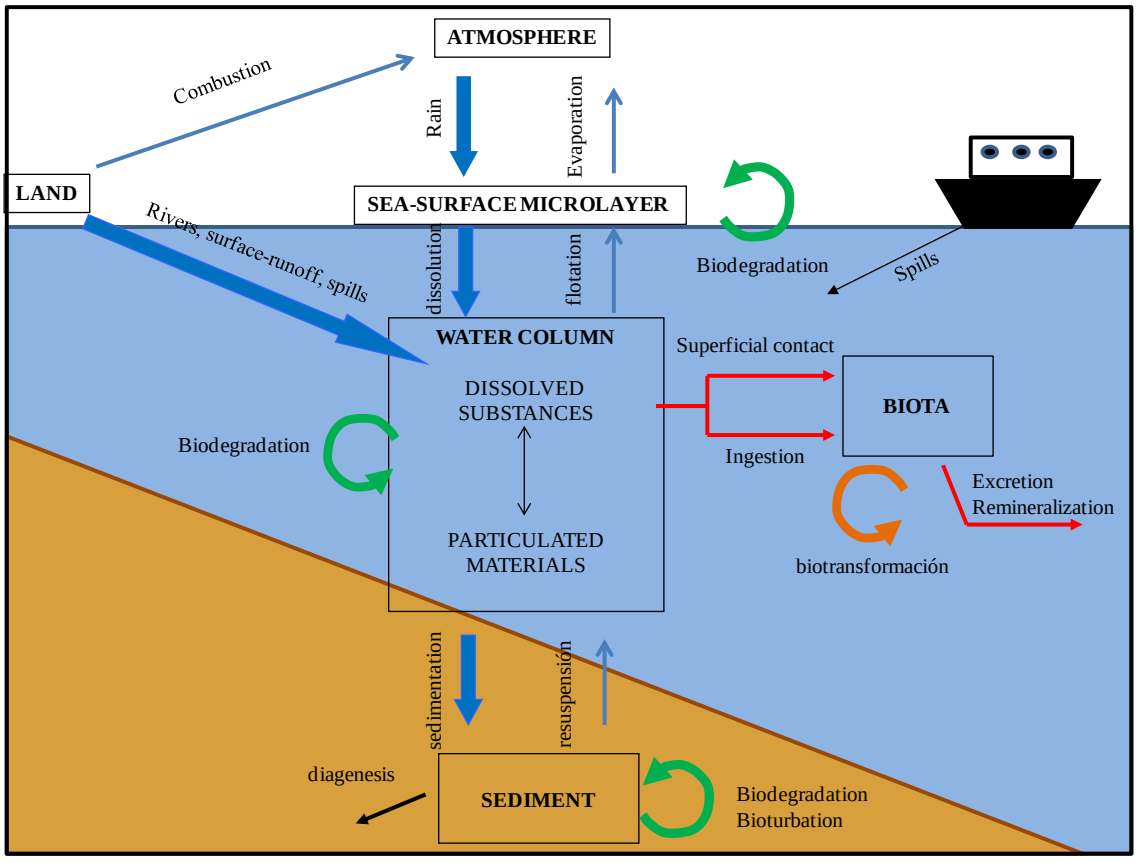


Figure 1. Schematic overview of pollution distribution in the marine environment and main processes involved. Boxes represent underwater matrices (water, sediment and biota). Size of arrows is proportional to the magnitude of the process.

Once a pollutant is accumulated by an organism, it can trigger different mechanisms. If the pollutant reaches the site of action, it can promote a toxic effect.

However, pollutants tend to be transformed by the metabolism in order to obtain less toxic substances and eventually be excreted. ***Biotransformation*** can be defined as the process whereby a substance is changed from one chemical to another (transformed) by a chemical reaction within the body (Timbrell, 2009). In the biotransformation process, organisms attempt to convert lipophilic substances into more polar, and consequently more readily excreted, metabolites. The exposure of the body to the compound is hence reduced and potential toxicity decreased.

When a substance cannot be, or it is hardly, metabolized will tend to accumulate in living organisms, reaching concentrations above those found in the surrounding environment. Thus, ***bioaccumulation*** can be defined as the concentration of a pollutant inside the organism that cannot be excreted. The bioaccumulation is dependent on the feeding activity and on the ability of organisms to assimilate each specific pollutant (Walker *et al*., 2009).

The ratio between the concentration of the pollutant inside the organism divided by the environmental concentration at steady state may be defined as the ***bioconcentration factor*** (BCF) , although some authors use this term for waterborne pollutants and use the term ***bioaccumulation factor*** (BAF) for pollutants incorporated via food (Walker *et al*., 2000). The steady state concentration results from a balance between uptake (from the water, the sediment, or food) and depuration (e.g. direct excretion, biotransformation) (Baumard *et al*., 1998). As a consequence, it is possible to identify different sites of interaction of a pollutant inside an organism (Figure 2). These are ***sites of action*** (when a chemical has a toxic effect on the organism), ***sites of metabolism*** (when the chemical is metabolized) and ***sites of storage*** (when chemical is not toxic and it is not metabolized).

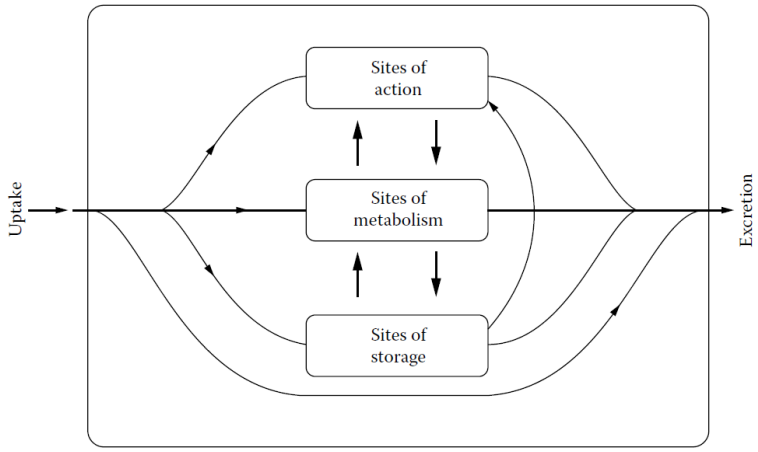


Figure 2. Schematic overview of the behavior of a foreign compound when it enters into an organism, interact with the different compartment and is excreted. Obtained from Walker *et al*., 2009.

3. How to measure marine pollution?

3.1. International and National marine regulations

As a consequence of the increasing concern about the threats of marine environmental pollution, numerous international organizations worked together for decades trying to elaborate an international legal framework to control and progressively eliminate pollution. The *United Nations Convention on the Law of the Sea* (ONU, 1982), established a basic framework to regulate the protection of the oceans. In Europe, for decades, the protection and conservation of the marine environment has been guaranteed by international agreements, such as the Regional Seas Conventions (RSC): the *Convention for the Protection of the Marine Environment in the North-East Atlantic* of 1992 (further to earlier versions of 1972 and 1974) – the ***OSPAR Convention*** (www.ospar.org), the *Convention on the Protection of the Marine Environment in the Baltic Sea* Area of 1992 (further to the earlier version of 1974) – the ***Helsinki Convention*** (HELCOM, www.helcom.fi), the *Convention for the Protection of Marine Environment and the Coastal Region of the Mediterranean* of 1995 (further to the earlier version of 1976) – the ***Barcelona Convention*** (www.unepmap. org), or the *Convention for the Protection of the Black Sea* of 1992 – the ***Bucharest Convention*** (www.blacksea-commission.org).

As a result of the interest in the protection of the marine environment, the European Commission enforced several environmental regulations for the protection of the marine environment, including the adoption of the ***Water Framework Directive*** (WFD; 2000/60/CE), which establishes a framework for the protection of groundwater, inland surface waters, estuarine (=transitional) waters and coastal waters. Nevertheless, the WFD only provided comprehensive coverage of a small part or the European marine waters. This led the European Commission and the member states to call for a new framework for European´s seas protection and preservation: the ***Marine Strategy Framework Directive*** (MFSD; 2008/56/CE). In Spain, the ***Law 41/2010 on the protection of the marine environment*** (LPME) is the standard transporting the MSFD into the national law, adapting the normative to the national scenario (Bellas *et al.*, 2014). In this sense, the Ministry of Agriculture, Food and Environment, MAGRAMA (http://www.magrama.gob.es) is the authority responsible for the implementation of the MSFD in Spain. The LPME keeps the general objective of the international normative, which consists of the conservation, protection and sustainable use of the marine ecosystems, with the main objective of achieving a *Good Environmental Status* (GES) of the marine environment by year 2020. The MAGRAMA coordinates the ***marine strategies*** in collaboration with the involved administrations; in this case, the Spanish Institute of Oceanography (IEO) which is the organism responsible for the surveillance of the quality and health of the marine environment in Spain.

3.2. Monitoring programs

The main tools to evaluate marine pollution are the ***monitoring programs***. A monitoring program can be defined as "*the systematic and continuous measurement of sources, levels and effects of chemical compounds along the time and the space using standardized methods of measure*" (WHO, 1974).

According to the World Health Organization (WHO, 1974), the surveillance o monitoring programs were designed:

- To manage environmental quality considering pollutants for which criteria and standards have been established
- To deal with pollution problems along wide geographic areas
- To determine pollution trends
- To facilitate retrospective assessment once a new pollutant or more sensitive technique have been identified
- To devise models of human exposure

The analysis of contaminants in monitoring programs has been addressed using different matrices:

Analysis of pollutants in water

Measurements of pollutants concentrations in water are sometimes complex and expensive since most pollutants are found in very small quantities in the water column and vary with time, especially in coastal waters, since they are dependent on the tides, the ocean currents, the wind or the intermittent flows. As a consequence, the information provided by chemical analysis of water reflects only the contamination at the moment in which the water was sampled.

Analysis of pollutants in sediment

Other abiotic compartment considered is the sediment. This matrix has been considered a better option for the evaluation of chemical pollution than water, since chemicals' concentrations found in sediments are higher and more stable in time, providing an integrated measure of pollution in a certain area. Moreover, this matrix is important in monitoring studies because acts as a sink for chemicals and also because it serves as a source of toxicants for marine organisms (US EPA, 2001)

Analysis of pollutants in biota

As stated above, when a chemical substance cannot be metabolized by the organism, it tends to be accumulated in their tissues, reaching higher concentrations that those found in the environment. In the case of marine organisms, toxicants are usually taken up through direct contact with body surfaces or through the ingestion of food. Consequently, the concentrations of pollutants in marine organisms are usually higher than those found in water, and also more stable over time (Wright and Welbourn, 2002).

Marine pollution monitoring can be based on the chemical analysis of a selected set of pollutants in environmental compartments: ***chemical monitoring***, but during last decades international organisms with competence in environmental management advocate also the measurement of effects in organisms caused by the exposure to pollutants: ***biological monitoring*** (Davies and Veethack, 2012). In fact, current monitoring programs use the combination of chemical and biological measurements in ***integrative monitoring schemes***, with the aim of establishing the link between the presence of pollutants in the environment and the effects on marine ecosystems. In addition, within the integrative monitoring, two approaches may be distinguished: ***active monitoring***, when organisms from a clean site are caged in certain areasto monitor and evaluate spatial and temporal patterns of chemical pollution and its effects, and ***passive monitoring***, when organisms from natural populations are sampled with the same objectives(Lacroix *et al*., 2015; Marigómez *et al*., 2013).

The first comprehensive effort to establish a monitoring program to assess the status of the coast was undertaken in the U.S. by means of the ***Mussel Watch Program***, initiated by the USEPA in the period 1976-78, and continued by the NOAA from 1986 until nowadays (Kimbrough *et al*., 2008). Mussel Watch Program was designed to monitor the status and trends of chemical contamination of the U.S. coastal waters, including the Great Lakes (Kimbrough *et al*., 2008). The Program is based on yearly collection and analysis of oysters and mussels and nowadays includes 300 monitoring sites where more than 140 chemical pollutants are measured. Many of these pollutants are included in the EPA Priority Pollutants list (Keith and Teillard, 1979).

In Spain, the ***Marine Pollution Group*** from the IEO has been working at the Oceanographic Centers of Vigo and Murcia for more than 30 years to assess pollution along the Spanish coasts, from a national and international point of view. In fact, the IEO carry out the Spanish monitoring routine established by the ***OSPAR*** and the ***Barcelona Conventions***. A schematic overview of the legal framework and national monitoring programs in Spain is provided in Figure 3. Briefly, ***Coordinated Environmental Monitoring Program*** (CEMP) framed in the *Joint Assessment and Monitoring Program* (JAMP) and the ***Program for the evaluation and control of pollution in the Mediterranean Region*** (MEDPOL) include, respectively, the recommendations and guidelines of the OSPAR Convention and the Barcelona Convention for monitoring marine pollution.

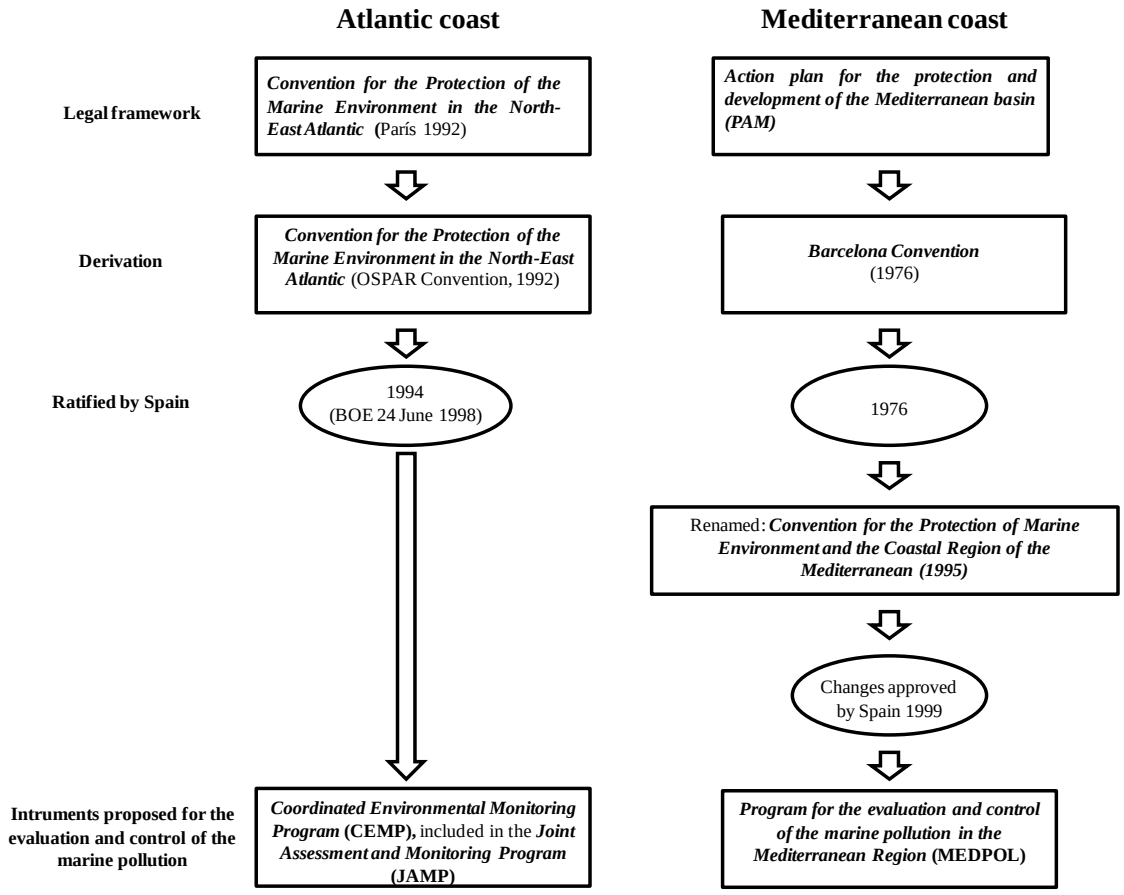


Figure 3. Diagram of the legal framework to control and evaluate marine pollution by international conventions, ratification by Spain and instruments used for the monitoring of marine pollution in the Spanish coast. Information from MAGRAMA web page (http://www.magrama.gob.es)

This framework has been adapted to elaborate the monitoring program of the MSFD descriptor 8 (Contaminants are at levels not giving rise to pollution effects), and thus articulate the commitments and obligations of the MSFD implementations with those of the Regional Seas Conventions. Since the MFSD's descriptor 8, demands an approach based not only on the analytical chemistry of pollutants but also on evaluating their effects on the ecosystems, marine pollution monitoring programs carried out in Spain (JAMP, MEDPOL) include the integrated use of both chemical analysis and biological techniques, in order to establish the link between pollutant levels and their harmful effects on marine ecosystems (Davies and Vethaak, 2012; ICES 2012; OSPAR Commission 2010; 2013). Table 1 shows the list of hazardous substances of prioritary concern considered by the WFD and by Regional Seas Conventions signed by Spain (OSPAR, Barcelona Convention).

Table 1. Hazardous substances of prioritary concern considered by WFD and by the Regional Seas Conventions signed by Spain (OSPAR and Barcelona Conventions). Information obtained from Law *et al*. (2010).

Pollutant	OSPAR	Barcelona Convention	WFD
Heavy metals and their compounds	**Cd, Hg, Pb in biota and sediment**	Hg, Cd, Pb, Zn, Cu, Cr, and their compounds. In addition: organometalic and, organolead compounds: tetramethyllead (TML) and tetraethyllead (TEL)	Hg, Cd, Pb, Ni and their compounds
Organotin compounds	**TBT in sediment and specific biological effects** TBT in biota as an alternative to monitoring TBT in sediments	Trialkyltin compounds	Tributyltin compounds
Chlorobenzenes		Mono-, di- and trichlorobenzenes	Tri-, penta-, hexachlorobenzenes
PCBs, dioxins and dioxins-like polychlorinated biphenyls	**PCB congeners (PCB28, PCB52, PCB101, PCB118, PCB138, PCB153 and PCB180 in biota and sediment** In biota: PCB77, PCB126 and PCB169. Also in sediment if congeners are 100 times higher than Background Assessment Concentration	PCBs (polychlorobiphenyles) and hexaclorobenzenes, dioxins and furans.	PCBs and dioxins are under review
VOC´s		Chlorinated solvents	Chloroform, Carbon tetrachloride, trichloroethylene,tetrachloroethylene, benzene
BFR	**PBDES and congeners (BDE28,47,66,85,99,100,153,154,183) in biota and sediment and also BDE209 in sediment**	PentaBDE and polybrominated biphenyls	pentabromodiphenylether (congeners28,47,99,100, 153and 154)
PFC	PFOS in sediment, biota and water		PFOS under review
Nonylphenol			Nonylphenol
Octylphenol			Octylphenol
Short-chain chlorinated paraffins		Chlorinated paraffins (CP) with carbon chain lengths of C10 to C30	Chloroalkanes, C10-C30
PAHs	**anthracene, benz [a] an-thracene, phenan-threne, fluoranthene, pyrene, chrysene, benzo [a] pyrene, indeno [1,2,3-cd] pyrene,benzo [ghi] perylene in biota and sediment** **C_1-, C_2-, C_3**-naphthalenes, C1-, C2-, C3-phenanthrenes, C1-, C2-, C3-dibenzothiophenes in biota and sediment, PAHs and metal-specific biologial effects	fluoranthene, benzo [a] pyrene, benzo [b] fluoranthene, benzo [k] fluoranthene, indeno [1,2,3-cd] pyrene,benzo [ghi] perylene	naphthalenes, anthracene, fluoranthene, benzo [b] fluoranthene, benzo [k] fluoranthene, benzo [ghi] perylene, indeno [1,2,3-cd] pyrene,
Organophosphorus compounds			Chlorpyrifos-ethyl
Organochlorine pesticides (DDTs) and other pesticides (HCHs)		Organohalogenated pesticides: gamma isomer of HCH, Chlorophenoxyacids Pesticides DDTs: aldrin, dieldrin, endrin, chlordane, heptachlor, mirex, toxaphene, hexachlorocyclohexane	aldrin, dieldrin, endrin, isodrin, DDT, p,p-DDT, hexachlorocyclohexane Endosulfan Herbicides: alachlor, trifluralin, atrazine, isoproturon, diuron, simazine. Under review for PS or PHS.
Chlorinated phenolic compounds		Chlorinated phenolic compounds mainly pentachlorophenol	pentachlorophenol
Radioactive substances		Radioactive substances	

***marked in bold substances under mandatory monitoring**

The species selected to satisfy the objectives of monitoring programs are recognized as ***bioindicators*** or ***biomonitors***. A bioindicator can be defined as an organism or group of organisms that reveals the presence of pollutants and allows characterizing the environmental status of an ecosystem in order to detect changes promoted by natural or anthropogenic stressors (Blandin, 1986). In this sense, molluscs and within them, mussels, are by far the most used organisms in monitoring programs to detect pollutant levels and their effects. Therefore, they have been strongly recommended as bioindicators by national and international institutions (Davies and Vethaak, 2012; Kimbrough *et al*., 2008), including conventions such as OSPAR, Barcelona and HELCOM conventions.

4. Why mussels?

The word "mussel" is most frequently refers to the edible bivalves of the marine family *Mytilidae*. Most of them live on exposed shores in the intertidal zone and are of great interest since they are intensively fished and cultured worldwide for human consumption (Dailianis, 2010).

Mussels of the genus *Mytilus* (Kingdom: *Animalia*, Phylum: *Mollusca*, Class: *Bivalvia*, Subclass: *Pteriomorphia*, Order: *Lamellibranchia*) are a group of filter-feeding bivalve molluscs, firstly recorded 1-2 millions years ago. Genetic analysis of mussels revealed 9 different species of the genus *Mytilus* spreading throughout the world (WORMS, 2016):

- *M. edulis*, (North hemisphere)
- *M. galloprovincialis*, (the common species in the Mediterranean Sea)
- *M. trossulus*, (coastal areas of North America, Pacific Ocean)
- *M. coruscus*, (coastal areas of China and Japan)
- *M. californianus*, (coastal areas of North America, Pacific Ocean)
- *M. chilensis*, (coastal areas of South America, Chile)
- *M. platensis*, (coastal areas of Argentina, South Atlantic Ocean)
- *M. planulatus*, (coastal areas of Australia)
- *M. desolationis*, (coastal areas of Kerguelen Islands, Indic Ocean).

Mussels were first used as indicator organisms to assess the spatial and temporal trends of chemical contamination in the *Mussel Watch* monitoring program in estuarine and coastal areas of North America (Goldberg, 1975) because of the following features:

- They are abundant in coastal communities and have a wide geographical distribution (WORMS, 2016).
- They are sedentary species and therefore, they show the chemical pollution in a given area. In addition, mussels are very resilient organisms and can be found in areas with high concentration of pollutants (Viarengo and Canesi, 1991; Regoli and Principato, 1995; Boening, 1999).
- They are suspension-feeders that pump large volumes of water and have a great capability to accumulate pollutants (Cranford *et al*., 2011; Widdows *et al*., 2002).
- Mussels have a relatively long life-cycle and their populations are relatively stable, which provide data on short and long-term temporal changes in contaminant levels (Kimbrough *et al*., 2008; Nakata *et al*., 2012; Sericano *et al*., 2014).
- They are easily collected and can be transplanted to an area of interest or maintained under laboratory conditions (Rodríguez *et al*., 2005).

5. What do we know about mussels?

5.1. Mussel anatomy

Mussels present an external shell covering their internal tissues. The mussel's external shell is composed of two ***valves***. Each valve has an elongated V-shape, starting at the anterior extreme or ***umbo*** and broadening out until the posterior extreme. The valves are mainly composed of calcium carbonate, growing from the umbo, generating several concentric rings which represent the mussel's age. Both valves are linked through a ***hinge*** that allows valves to close and perfectly seal. The valves are joined together on the outside by a ***ligament,*** and are closed by the action of two muscles (anterior and posterior). Mussel shells carry out a variety of functions, including support for soft tissues, protection from predators and protection against desiccation (Gosling, 2003). From the external part of the mussels (shell) to the intern part, it is possible to distinguish: ***mantle, gonads, gills*** and ***visceral mass.***

The mantle consists of two lobules of connective tissue that envelop the rest of the soft tissues keeping them linked to the shell. In addition, it contains hemolymph vessels, nerves and muscles that are particularly well developed in the mantle margin, the ***mantle edge***. The mantle is the main site for the storage of energetic reserves but it also contains most of the ***gonad*** structure (Crespo and Espinosa, 1990). The gonad is not a different organ from the mantle but it consists of branched tubules that invade the mantle ending in a genital follicle formed by gonias and follicle cells (Lubet, 1959). Close to the mantle, mussels have the ***gills,*** the principal organs of respiration and food capture (Bayne *et al*., 1976). Gills are lamellae structures which consist of four pairs of demibranchs, which are, in turn, composed by two lamellae (descending and ascending)

that have long filaments with ciliated areas responsible to create water flows called ctenidia (Filgueira, 2007). Moreover, these ctenidia have three ciliated areas which differ in their biological function: the lateral cilia are responsible of the water movement through the ostia (space between ctenidia), the frontal cilia transport the filtered particles to the palps-mouth system and laterofrontal cilia are responsible to subtract the food particles from the water flow (Babarro *et al*., 2000).

Within the visceral mass it is possible to distinguish: ***foot***, ***byssal gland*** and the ***digestive*** and ***circulatory systems***. The digestive system includes the ***mouth***, ***oesophagus***, ***stomach***, ***digestive gland*** and ***anus***. The mouth is a ciliated structure that leads into a narrow ciliated oesophagus. The stomach is large and is completely embedded in the digestive gland. A semi-transparent gelatinous rod, called crystalline style, is originated at the end of the stomach and has the ability to rotate against the gastric shield. The style has additional functions in the digestive process. In the process of rotation, the style end is dissolved releasing carbohydrate-splitting enzymes in the process (Bayne *et al*., 1976). In addition to the role of food digestion, the digestive gland of mussels serves as a site to storage metabolic reserves, which provide a source of energy utilized during gametogenesis and during periods of physiological stress (Thompson and Bayne, 1974). Rejected particles from the stomach and waste material from the digestive gland pass into the long coiled intestine and are transformed to faecal pellets before being thrown away in the anus (Gosling *et al*., 2003).

The ***foot*** is a long and highly mobile muscular structure located close to the byssal gland. Mussels secrete byssal threads to better attach to the substrate (Bayne *et al*., 1976). Finally, mussel´s circulatory system consist on hemolymph,free flowing through the different tissues and a small heart (Nicholson, 2002). The heart consists of a single, muscular ventricle and two thin-walled auricles. Hemolymph flows from the auricles' in the ventricle, which contracts to drive the hemolymph into a single vessel, the anterior aorta. The anterior aorta divides into many arteries that supply the mantle, stomach, intestine, shell muscles and foot (Gosling *et al*, 2003).

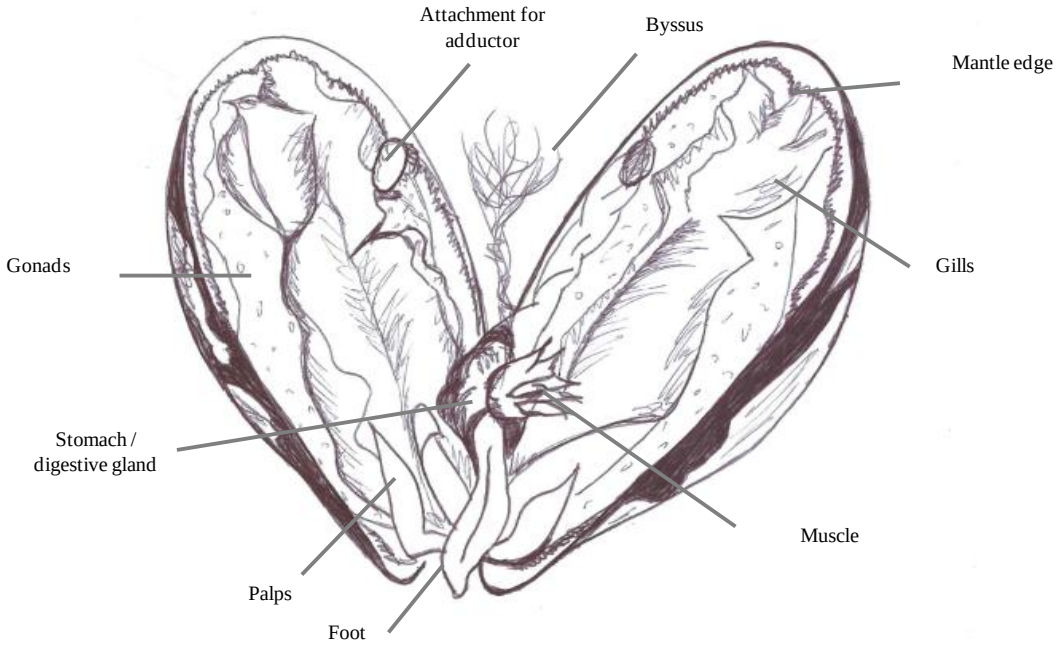


Figure 4. Anatomical structure of mussels *Mytilus spp*.

5.2. Mussel physiology: Feeding and Metabolism

Bivalves are sessile filter-feeders, which mean that they obtain their energy filtering the suspended particles (seston) from the water. This suspended particulate matter is quite heterogeneous ranging from inorganic to organic particles. Particulate organic matter is composed by living organisms as phytoplankton, detritus, microorganisms and faecal pellets (Lucas, 2008). As they live in littoral and shallow sub-littoral waters which are characterized by a high spatial and temporal variability in terms of food quantity and quality (Newell, 1979), they have developed several mechanisms to select high quality particles in order to optimize energy intake (Ward and Shumway, 2004).

As mentioned above, gills are the main organ responsible for filtration. Once particles are filtered, they are transported to the palps and finally the mouth, where they are ingested. Particle selection can take place at different feeding stages: selective retention in the gills, pre-ingestive sorting in the palps and post-ingestive sorting in the digestive gland (Ward and Shumway, 2004), as shown in Figure 5.

Particles flowing through the gills are retained by the frontal surfaces of the ctenidia which act as a sieve. ***Particle capture*** is dependent mainly on particle size (Defossez and Hawkins, 1997; Lehane and Davenport, 2006), although other qualitative factors have also been considered: shape (Bougrier *et al*., 1997), nutritive value (Prins *et al*., 1991; MacDonald and Ward, 1994; Hawkins *et al*., 1996; 1998) or chemical composition of the surface of the particle (Yahel *et al*., 2009). Observations of mussel

filtering in natural seawater indicate that they can filter a particle range from 2-100 µm (Bayne *et al*., 1976). Recent studies have deepen into this knowledge, demonstrating that mussels have higher retention efficiency for particles within a range of 30 to 35 µm compared to smaller particles of 1 to 15 µm diameter (Strohmeier *et al*., 2012).

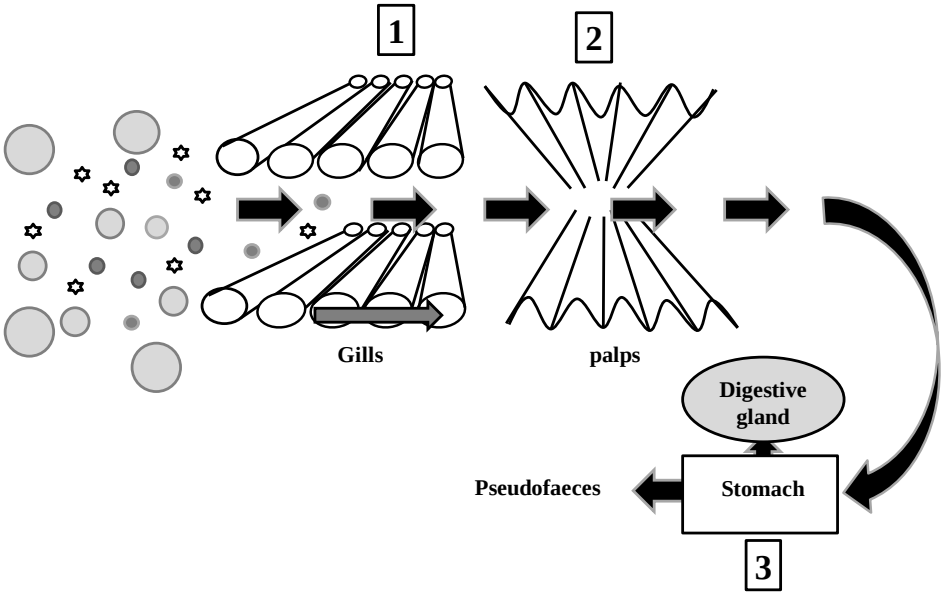


Figure 5. Diagram of the particle selective processes in bivalves: (1) selective retention in the gills, (2) pre-ingestive sorting in the palps and (3) post-ingestive sorting in the digestive gland. Arrows indicate general movement of particulate matter from the environment (left) through the feeding and digestive organs. Adapted from (Ward and Shumway, 2004).

The most commonly used measurement of filtering activity is the ***clearance rate,*** which is defined as the volume of water completely cleared of particles per unit of time. This term is sometimes confused with ***filtration*** or ***pumping rate***, which is the total volume of water flowing through the gills per unit of time. If 100% of particles are removed from the water, filtration rate and clearance rate have the same numerical value. Mussels are able to filter great volumes of water ranging from 0.5 to 7 liters hour according to size (Santiago, 2007), being filtration one of the most important physiological processes in bivalves. This encouraged the emergence of two opposite theories on regulation of filtration in bivalves. The first one, led by Jørgensen, considered that feeding processes were dependent on the species and assumed that particles selection depend only on gill structure and particle concentration in the surrounding water (Jørgensen, 1996). The opposite theory, led by Bayne, assumed that filtration was a physiologically controlled process which depended on the nutritional needs of the organism and on the qualitative and quantitative composition of the seston (Bayne, 1998). This theory presents suspension feeding as a complex interaction between physiological, morphological and behavioral characteristics which are sensitive to the variation of available food in the environment.

The filtration process can be influenced by several biotic and abiotic factors (Ackerman and Nishizaki 2004; Camacho, 1991; Filgueira *et al*. 2009; Iglesias *et al*., 1992; Neira *et al*., 1990; Velasco and Navarro 2002). One of these factors is the mussel

body size. It has been described that there is an inverse relationship between the weight-specific filtration rate and mussel length or body weight (Thompson and Bayne, 1974) which is related to a reduction of the ratio (gill area)/(dry weight of the animal) in larger animals. Thus, considering that filtration rate is a function of body size which follows the allometric equation: Filtration=a × $Size^b$ (*b* always shows values <1) (Cranford *et al*., 2011).

In the same way, it has been described that bivalves have the ability to regulate the filtration rate according to the concentration of the particles available in the water column (Winter, 1978; Widdows, 1978; Bayne and Newell, 1983; Albentosa *et al*., 1996; Hawkins *et al*., 2001; Velasco and Navarro, 2003). This regulation pattern is as follows: when food concentration is very low, filtration increases rapidly and is kept constant up to the food concentration at which maximum ingestion is reached, which is also the optimum ingestion for digestive purposes. After this maximum, filtration decreases in order to maintain constant this optimum ingestion. If concentration is still increasing, not only filtration but also ingestion decreases by the production of pseudofaeces (particles which have been filtered but not ingested) (Winter, 1978).

Once the particles have been captured in the gills, they are transported to the labial palps where ***pre-ingestive selection*** occurs. Particle sorting leads to the formation of pseudofaeces which are rejected to the paleal cavity and removed through the exhalant current (Ward and Shumway, 2004). At this level, it has been described that palps can discriminate between organic and inorganic particles, rejecting the inorganic ones as pseudofaeces (Lucas, 2008) and increasing the organic fraction of the ingested food. Thus, pseudofaeces production is a mechanism that tend to improve energy intake by means of both, maintaining constant maximum ingestion and selecting high-quality particles (Iglesias *et al*., 1992).

Particles accepted by the labial palps pass through the digestive system. Two different types of digestion can be differentiated: ***extracellular digestion***, which takes place in the stomach by enzymes released from the rotating crystalline style, and ***intracellular digestion***, which occurs immediately after the stomach in the digestive gland. The unabsorbed material from the digestive gland and the stomach is formed into faecal pellets that are directly released through the anus (Gosling, 2003). The above mentioned mechanism of ***post-ingestive selection*** takes place in the stomach after the action of extracellular enzymes and the mechanical action of the crystalline style on ingested food. At this point, the most nutritious particles (lighter organic particles) are directed into the ducts of the digestive gland whereas denser inorganic material passes into the intestine and is incorporates into faecal pellets (Ward and Shumway, 2004).

Digestive capacity is a function of the combination of digestive parameters such as the time that food resides in the digestive system (gut retention time) and the total amount of food inside the digestive (gut capacity), which depend on the physical

properties of the gut, and the digestive enzymes activity. Therefore, ***absorption efficiency*** can be defined as the proportion of the organic matter ingested that is absorbed. Absorption depends on the quality and quantity of the food ingested. Some authors have postulated that the absorption efficiency could be also regulated according to the nutritional necessities (Winter, 1978; Widdows *et al*., 1979; Beiras *et al*., 1993). This is a complex process which involves the clearance and ingestion rates, which are adjusted in such a way to maintain constant the ingestion and as a consequence, the food is to be absorbed with equal efficiency. At high food concentrations, the ingestion ration increases and the digestion capacity of the digestive gland is exceeded, resulting in a high proportion of food rejected in the faeces with the associated decrease of the absorption efficiency (Griffiths and Griffiths, 1987).

Gills serve as a dual function, respiration and feeding. They are responsible for the gas exchange due to its high surface area and rich supply of hemolymph. In this sense, large volumes of water pass through the gill on whose surface takes place the extraction of oxygen that diffuses into the hemolymph (Bayne *et al*., 1976; Gosling, 2003). The most usual method to measure ***respiration rates*** is the measurement of the oxygen consumption per unit of time. As it has been described for filtration and absorption, respiration could also be affected by some biotic and abiotic factors such as mussel body weight (size) or temperature (for a review see: Winter, 1978; Bayne and Newell, 1983; Jørgensen, 1990; Riisgard, 2001). As a brief summary, decreases of weight-specific respiration rates with size have been reported in *M. edulis* (Sukhotin and Pörtner, 2001) and, to a lesser extent, in *M. galloprovincialis* (Pérez Camacho *et al*., 2000). Since mussels are sessile animals, other factor influencing respiration is related to the mussel activity level defined as filter-feeding activity. According to feeding activity, it two components of the metabolic rate can be established: i) the standard metabolic rate which is observed in starved or poorly-fed mussels and, ii) the active metabolic rate which is observed in well-fed animals, and which includes the costs of water transport, filtration, digestion and absorption (Bayne and Newell, 1983). The ability of bivalves to regulate metabolic costs according to food availability, together with regulation of feeding, as described above, have substantial adaptive significance in the variable intertidal environment where they live (Griffith and Griffiths, 1987).

5.3. Mussel immunology

Bivalves have an open circulatory system. The hemolymph consists of cells with colourless plasma that plays a number of important roles in bivalve physiology including gas exchange, osmoregulation, nutrient distribution, waste elimination and internal defence (Gosling, 2003). The hemolymph contains cells called haemocytes that were classified by Cheng (1981) based on a morphological criteria as: ***hyalinocytes***, small and agranular cells, and ***granulocytes***, actively phagocytic cells containing numerous acidophilic cytoplasmic granules.

The bivalve immune system is considered non-specific, which means that it provides immediate defence recognizing and responding to noxious particles and

environments in a generic way. In comparison to other bivalves, mussels show a higher level of immunological defence probably linked to their resilience to variable environmental conditions (Wootton *et al*., 2003). Since mussels have an open circulatory system, hemocytes can be concentrated in different tissues (gills, digestive gland and muscle). ***Hemocyte viability*** (percentage of dead hemocytes from the total of live cells) can be an indicative of mussel immune status (Hegaret *et al*., 2003). However, the predominant mechanism of internal defence in bivalves involves ***phagocytosis*** or ***encaptulation*** by the circulating haemocytes. Phagocytosis is the ability to recognize and ingest non-self molecules and cell debris. The first step of phagocytosis is the attachment of the phagocyte to the targeted particle (Gosling, 2003). Many studies have revealed that hemocytes of many bivalves exhibit chemotactic as well as chemokinetic reactions, depending on the nature of the molecules. In *M. galloprovincialis*, both types of hemocytes could execute phagocytosis by formation of coated vesicles and uncoated endocytic vesicles. After that, phagosomes and lysosomes fuse together and the engulfed target is destroyed within phagosomes by lysosomal enzymes, reactive oxygen species (ROS) and nitric oxide (NO), as well as by antimicrobial factors. The release of degradative enzymes for the destruction of foreign material is accomplished by a sudden release of ROS within hemocytes which is referred to as ***respiratory burst***. ROS act as killing agents, either alone or in combination with lysosomal enzymes, and are important in the phagocyte-mediated killing microorganisms (Song *et al*., 2010). Successful elimination of potential infective agents requires granulocytes to engulf particles, and to eliminate living pathogens by enzymatic or oxidative degradation. The hemocytes are able to recognize and to phagocytise foreign particulate matter such as microorganisms, carbon particles and foreign protein molecules (Song *et al*., 2010).

5.4. Mussel reproduction

Bivalves are characterized by having a simple reproductive system. Gametogenesis is carried out in the gonads and they have an external fertilization in which gametes are released into the water (Cáceres-Martínez and Figueras, 2007). Females develop oogonias (female sacs) and males develop spermatogonias (male sacs) (Bayne *et al*., 1976).

In general, an annual reproductive cycle has been described for *Mytilus spp.* according to which gametogenesis takes place between late autumn and winter until the beginning of the spring, when the gonad is completely ripe and spawning occurs (Seed, 1976; Bayne, 1976, 1984; Lubet, 1959). However, this general pattern differs grately within the same population depending on latitude and on food availability, and also between years.

Mussel reproductive cycle comprises the entire cycle of events from activation of the gonad, through the gametogenesis to spawning (release of gametes) and the subsequent recession of the gonad. It can be divided into ***reproductive stages*** or ***periods***, which start with the initiation of gametogenesis and culminate with the emission of

gametes, and the *vegetative* or *resting stage*. According to Kim *et al*. (2006), it is possible to distinguish between 5 different reproductive stages (Figure 6): Stage 0, inactive gonad; Stage 1, gametogenesis has begun although no ripe gametes are visible yet; Stage 2, ripe gametes are present and gonias occupy about one-third of the section ; Stage 3, gonad increased in area to about half of the fully ripe condition ; Stage 4, fully ripe gonad .

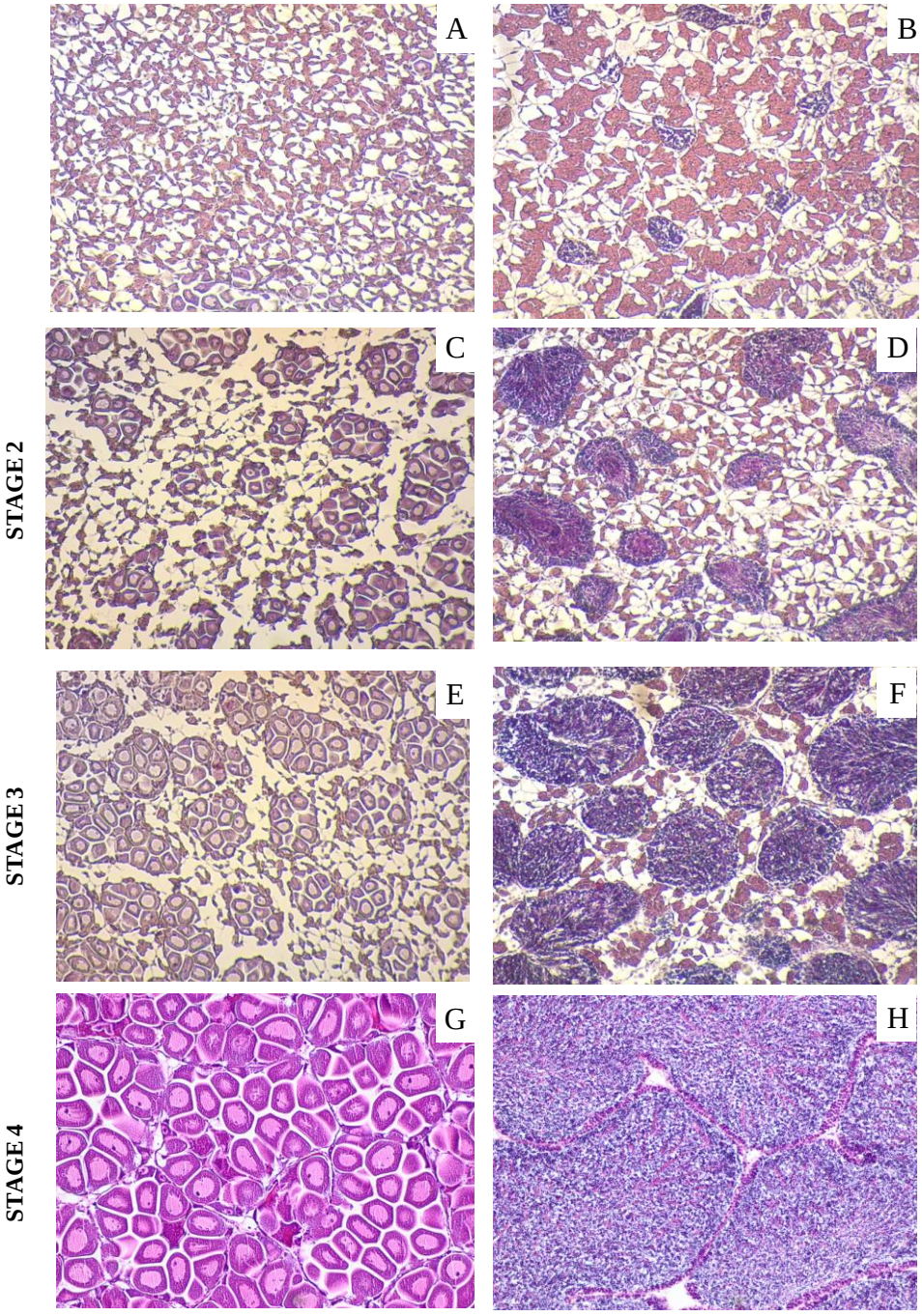


Figure 6. Histological section of mussel gonad stained with hematoxylin/eosin. (A)Stage 0; (B) Stage 1; (C) Stage 2, female; (D) Stage 2, male; (E) Stage 3, female; (F) Stage 3, male; (G) Stage 4, female; (H) Stage 4, male.

Furthermore, mussel´s reproductive cycle is characterized by a parallel annual storage cycle. Gametogenesis is an energy demanding process. During the annual reproductive cycle, nutrients are stored when food is abundant and gonad activity is minimal. Subsequently, these reserves are utilized to meet the energetic requirements of gametogenesis. The mantle is the main site for energetic reserves storage such as carbohydrates, lipids and proteins (Crespo and Espinosa, 1990). Most of the studies show that changes in body weight are mainly caused by changes in carbohydrate, or glycogen, which is the main source of carbohydrates. The seasonal cycles for storage and utilization of glycogen reserves are closely linked to mussel´s reproductive cycle and are strongly dependent of food supply and temperature (Gabbot and Bayne., 1973).

5.5. Toxicity and damage mechanisms

5.5.1. Reactive oxygen species (ROS)

A wide range of contaminants can promote the generation of free radicals, notably oxygen free radicals, also known as ***reactive oxygen species*** (ROS) or ***reactive oxygen intermediates*** (ROI). This term, includes not only the superoxide anion (O_2^-) or the hydroxyl radical (OH^-), but also other chemical derivatives of O_2 such as hydrogen peroxide (H_2O_2) (Di Gulio *et al*., 1989). The elevation of ROS production caused by the exposure to pollution can occur by several mechanisms such as the uptake of redox cycling metals and organic pollutants, the metabolism of pollutants to redox cycling derivates and the induction of oxyradicals generating enzymes (Livingstone *et al*., 1989). For example, organic compounds can generate ROS during the Quinone´s redox cycle or by oxidation through oxygenase enzymes such as CYP-450. H_2O_2 is transformed in OH^- via *Fenton reaction* using a metal as catalyst (commonly iron) (Reactions 1,2) whereas *Haber Weiss reaction* generates OH^- after the reduction of an oxidized metal by O_2^- and its reaction with H_2O_2 (Reaction 3) (Regoli and Giuliani, 2014):

Reaction 1 $$Metal^{n+1} + O_2^- \rightarrow Metal^{n+} + O_2$$

Reaction 2 $$Metal^{n+} + H_2O_2 \rightarrow Metal^{n+1} + OH^- + OH^-$$

Reaction 3 $$O_2^- + H_2O_2 \rightarrow O_2 + OH^- + OH^-$$

On the other hand, not only pollutants can generate ROS production but also they are naturally produced during several cellular pathways of aerobic metabolism including oxidative phosphorylation, electron transport chains or even immunological reactions as phagocytosis (Pipe *et al*., 1990).

5.5.2. Lipid membrane peroxidation (LPO)

Cellular membrane´s stability is essential to develop the vital function of cells. Cellular membranes are constituted by two lipidic layers which are rich in polyunsaturated fatty acids (PUFAs). PUFAs are especially vulnerable to oxidation by ROS, since ROS are more soluble in the fluid lipid bilayer than in the aqueous solution (Moller at al., 2005; Gamliel *et al*., 2008).

The overall process of lipid peroxidation consists of three steps: initiation, propagation, and termination. In the first step (initiation), ROS molecules, generally OH^-, react with hydrogen atoms (H^+) from the lipidic membrane creating an unstable lipidic radical (L^-) (Reaction 4). In the propagation phase, lipidic radicals can be linked to oxygen molecules forming lipid peroxide radical (LOO^-) (Reaction 5) which is still unstable. Finally, in the termination phase, this peroxide radical can react with a lipid, generating a new lipid hydroperoxide ($LOOH^-$) and an unstable lipid radical (L^-), promoting a cyclic process (Reaction 6). A comprehensive review regarding the chemistry associated with each of these steps is available elsewhere (Yin *et al*., 2011).

Reaction 4 $L^-H + OH^- \rightarrow L^- + H_2O$

Reaction 5 $L^- + O_2 \rightarrow LOO^-$

Reaction 6 $LH + LOO^- \rightarrow L^- + LOOH^-$

The membrane lipid peroxidation quantification, or lipid peroxidation, (LPO) can be described generally as a process under which oxidants, such as free radicals or non-radical species, attack lipids containing carbon-carbon double bond(s). In particular that occurs with PUFAs that involve hydrogen abstraction from a carbon, with oxygen insertion resulting in lipid peroxide radicals and hydroperoxides (as described before). LPO indicates the damage to cellular membrane lipids caused by ROS and is useful for assessing exposure to, and the effects of, pollutants in mussels (Campillo *et al*., 2013; Cheung *et al*., 2001; ICES, 2013; Regoli and Principato, 1995).

5.6. Mussel detoxification system

5.6.1. Biotransformation

Biotransformation has been defined as the process whereby a substance is changed from one chemical to another (transformed) by a chemical reaction within the body (Timbrell, 2009). Two major groups of enzymes are involved in the biotransformation processes, in which xenobiotics are converted into more polar and, consequently, more readily excreted metabolites (Figure 7): phase I and phase II enzymes (Van der Oost., 2003).

5.6.1.1. Phase I biotransformation process

Phase I enzymes are the responsible of *functionalization reactions*. During phase I metabolism, the foreign compound is chemically modified (e.g. oxidation, reduction or hydrolysis) to obtain a reactive group for the biotransformation in phase II (Livingstone., 1991). The most important phase I enzyme described in mussels is the cytochrome P450 monooxygenase (CYP), which forms part of the mixed function oxygenase (MFO) system (Snyder *et al*., 2000).

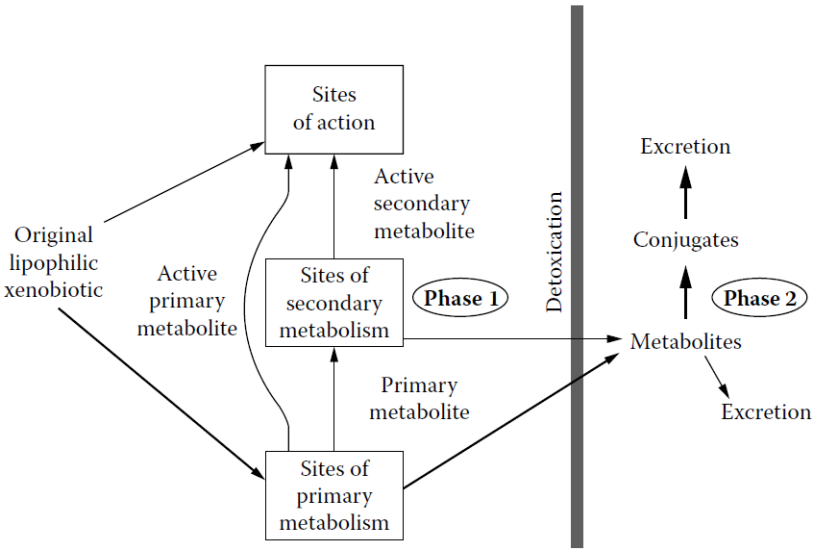


Figure 7. Diagrammatic representation of xenobiotic metabolism and processing in the body based on the biotransformation (phase I and II) processes. From Walker *et al*., 2009.

5.6.1.2. Phase II biotransformation process

Phase II biotransformation processes are considered as *conjugative processes.* In this phase, metabolites produced during phase I are linked to water soluble endogenous compounds naturally present in the cells (Livingstone, 1985). Linkage metabolites produce substances with higher water solubility facilitating their elimination by excretion. In some cases, non-enzymatic conjugation can occur. It is the case of some polar xenobiotics, that can be biotransformed directly through conjugation with parental

compounds without the prior phase I metabolism (Figure 7). Glutathione S-transferases (GSTs), sulfo-transferases, and other phase II enzymes allow the excretion of metabolites from phase I of biotransformation via specific trans-membrane transporters. The most important enzyme in this phase is the GST which consists in dimeric proteins formed by two subunits, identical or different, having each one an independent catalytic activity. GST is a catalyst in the glutathione (GSH) conjugation reactions of metabolites which normally come from the phase I. This enzyme is involved in the conjugation and detoxification of organic compounds, and also plays a protective role against oxidative stress by catalyzing a selenium-dependent glutathion peroxidase (Se-GPx) (Sheehan *et al.*, 2001).

5.6.2. Antioxidant defences

5.6.2.1. Non-enzymatic antioxidant defences

Although specific enzymes are responsible for ROS elimination, there is also non-enzymatic activity involved in the elimination of the highly reactive radicals, such as hydroxyl radicals (Brunk and Cadenas, 1988). In fact, ROS can be neutralized through small molecules recognized as ROS scavengers. Scavengers neutralize ROS by direct reaction with them, thus being temporarily oxidized before being reconverted by specific reductases to the active form. Scavengers can act as antioxidants in the cytoplasm or are intended to arrest the propagation of lipid peroxidation reactions on the membranes (Regoli and Giuliani, 2014). These can be hydrophilic molecules (e.g: GSH, vitamin C or ascorbate) or lipophilic molecules (e.g: vitamin E, vitamin A or carotenoids), whose levels have been shown to be elevated under oxidative-stress situations (Anderson *et al*., 1988).

In this sense, one of the most abundant cytosolic scavengers is reduced glutathione (GSH), which directly neutralizes several reactive species through its oxidation to oxidized glutathione (GSSG). In addition, GSH acts as a cofactor of several antioxidant glutathione-dependent enzymes (Regoli and Winston, 1998).

5.6.2.2.. Antioxidant enzymatic defences

Compared to scavengers, which interact with more than one type of ROS, enzymatic antioxidants catalyze highly specific reactions with specific substrates; however, these reactions still appear closely connected through several pathways (Figure 8).

Superoxide dismutase (SOD)

The enzyme superoxide dismutase (SOD), is the enzyme responsible for the removal of superoxide radical (O_2^-) with formation of hydrogen peroxide (H_2O_2) (Reaction 7). It has an essential antioxidant role since O_2^-, is the most abundant ROS in the cell. The radical O_2^- is generated through several metabolic processes such as the

oxidation of endogenous molecules or the oxygenases and deshydrogenases activity (Winston and Di Giulio, 1991). Different SOD isoforms are distinguished depending on their active center, acting as catalyst converter of the O_2^- dismutation reaction. Mn-SOD is localized within mitochondria, while Cu/Zn-SOD is found in the cytosol and peroxisomes (Nordberg and Arnér, 2001).

Reaction 7 $O_2^- + O_2^- \rightarrow H_2O + O_2$

Catalase (CAT)

The enzyme catalase (CAT) is a hemoprotein formed by four monomers of 60 kDa. In aerobic organisms it is the main catalytic route of reduction of H_2O_2 to H_2O (Reaction 8), working at high levels of H_2O_2, but at low levels it modulates the detoxification of other substances as phenols and alcohols through reactions coupled to the H_2O_2 reduction (Reaction 9). The CAT activity takes place mainly in peroxisomes, which are cellular organelles where cholesterol and fatty acid oxidation takes place, generating H_2O_2 as a by-product (Stegeman *et al*., 1992).

Reaction 8 $2H_2O_2 \rightarrow 2H_2O + O_2$

Reaction 9 $ROOH^- + AH_2 \rightarrow H_2O + ROH + A$

Glutathione peroxidase (GPx)

The glutathione peroxidase (GPx) acts as a catalyst in the reduction of H_2O_2 to H_2O, as well as CAT, but using reduced glutathione (GSH) as electron donor (Reaction 10). Moreover, this enzyme catalyzes the reduction of hydroperoxide ($ROOH^-$) to its corresponding alcohol (ROH) (Reaction 11), using NADPH as electron donor (Reaction 12). GPx enzymes are localized in the cytosolic and mitochondrial subcellular fractions. Although several isoforms have been described, the most abundant GPx isoforms are the selenium dependent GPx (Se-GPx), which are enzymes of 84 kDa, constituted by four sub-units, that contains one atom of selenium in its active center (Ursini *et al*., 1985). On the other hand, the non-selenium dependent GPx isoforms (non-Se-GP) correspond to glutathione S-transferase enzymes with a GPx function. They consist in small dimmers of 50 kDa, located mainly in cellular microsomes which only calalyze the reduction of organic peroxides (Lawrence *et al*., 1978).

Reaction 10 $H_2O_2 + 2GSH \rightarrow GSSG + 2\ H_2O$

Reaction 11 $ROOH^- + 2GSH \rightarrow ROH + GSH + H_2O$

Reaction 12 $GSSG + NADPH + H^+ \rightarrow GSH + NADP^+$

Glutathione reductase (GR)

Glutathione reductase (GR) is a dimeric flavoenzyme which catalyzes the reduction of GSSG in two molecules of GSH (Reaction 13), through a simultaneous reaction of oxidation of NADPH. This enzyme is the responsible to maintain the correct GSH/GSSG ratio and the intracellular redox status in marine organisms (Manduzio *et al.*, 2005). The reaction catalyzed by GR requires NADPH as an essential cofactor; this reducing equivalent is produced via the pentose phosphate cycle by glucose-6-phosphate dehydrogenase (G6PDH) which, similarly to GR, can be considered as an additional component of the antioxidant system (Regoli and Giuliani., 2014)

Reaction 13 $GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$

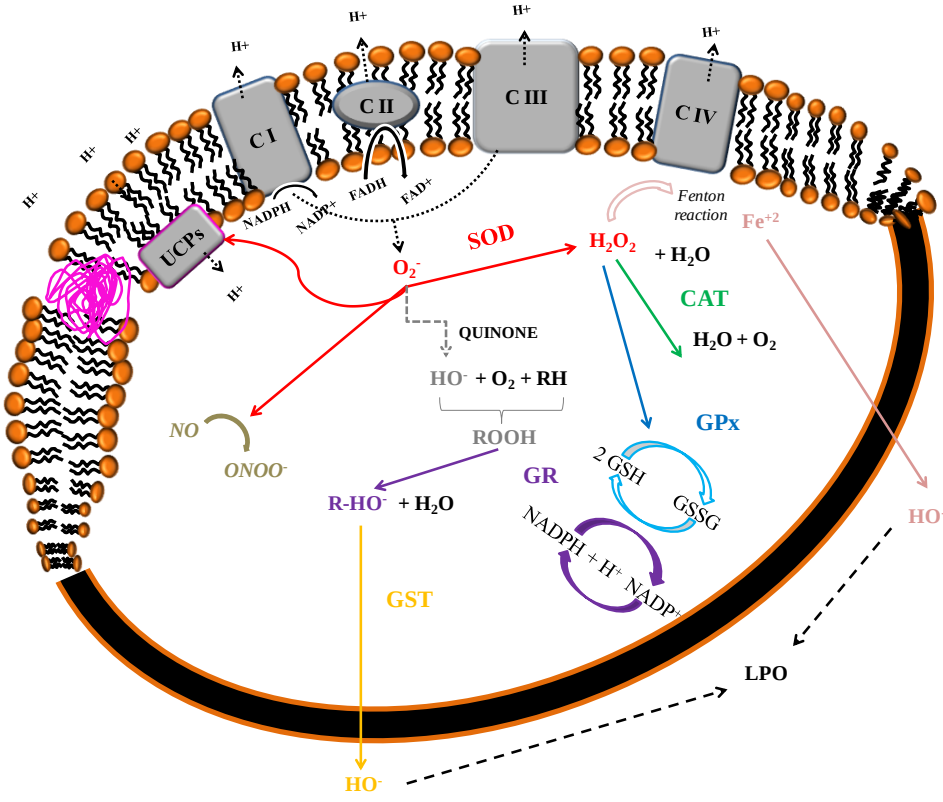


Figure 8. Main enzymatic defences of the cell against ROS. SOD: Superoxide dismutase, CAT: Catalase, GPx: Glutathion peroxidase, GR: Glutathione reductase, GST: Glutathione-S-transferase. LPO: lipid membrane peroxidation. Information extracted from Pamplona and Constantini, (2011).

6. How can the effects of pollution be quantified?

Understanding the uptake, intracellular transport processes and fate of chemicals, as well as their biotransformation and elimination, is essential to provide information about potential health risks of environmental pollutants for exposed organisms. Moreover, this knowledge helps to identify suitable *biomarkers* for use in marine pollution monitoring studies. ***Biomarkers*** can be defined as quantitative measurements of changes occurring at cellular, biochemical, molecular, or physiological levels, that can be measured in cells, body fluids, tissues or organs within an organism, and that may be indicative of xenobiotic exposure and/or effect (McCarthy and Shugart, 1990; Lam and Gray, 2003). According to that, biomarkers can be classified as (Van der Oost *et al*., 2003):

- *Biomarkers of exposure:* covering the detection and measurement of an exogenous substance, or its metabolites, or the product of an interaction between a xenobiotic agent and some target molecule or cell, that is measured in a compartment within an organism.
- *Biomarkers of effect*: those associated with an established or possible health impairment or disease.
- *Biomarkers of susceptibility*: indicating the inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance. That includes genetic factors and changes in receptors which alter the susceptibility of an organism to that exposure.

One of the key function of biomarkers is to provide an early signal of significant biological effect. It is generally believed that sub-organismic (molecular, biochemical and physiological) responses precede those that occur at higher levels of biological organization (Lam., 2009). Despite the fact that biomarkers are considered useful tools to measure the effects of pollution exposure, there is a general agreement that measuring a suite of indicators across different levels of biological organization is often necessary to assess ecological integrity (Adams *et al*., 1992; Clements, 2000).

As consequence, in this thesis, biomarkers at different levels of biological organization have been considered for a better interpretation of pollution effects on mussels. Figure 9 was adapted from Walker *et al*, (2000) including only the different biological responses considered in this thesis to identify the effects of pollution in mussels. A perspective from biochemical to whole organism responses is used to obtain a broadest view of the effect of pollution in mussel biology.

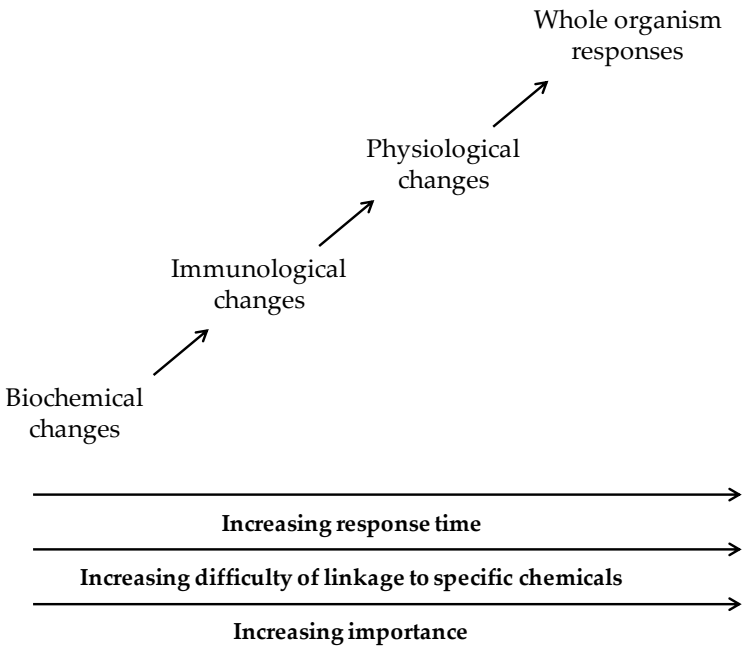


Figure 9. Schematic relationship of responses at different levels of biological organization. Adapted from Walker *et al*., 2000.

6.1. Immunological biomarkers

The increased observation of pollution-induced disease conditions in marine organisms has led to a growing interest on the effects of environmental contaminants on the immune system (Renault, 2015). Bivalves filter large volumes of water and their immune capacities can be adversely affected by exposure to contaminants. The condition of the immune system of marine bivalves partly determines their susceptibility to disease and survival. Therefore, the measure of endpoints linked to immunity can help identify sub-lethal effects of exposure to contaminants and provide early-warning signals (Gosling, 2003). The modulation of bivalve immune system has been altered in presence of several pollutants such as PCBs (Gagnaire *et al*., 2007; Geret *et al*., 2013). Specifically in mussels, it has been reported quick immune response in cell viability and in phagocytic capacity in presence of azamethiphos for short periods, up to 24 h (Canty *et al*., 2007). Other studies evidenced a modulation of the immune system in bivalves exposed to heavy metals (Auffret *et al*., 2002; Balbi *et al*., 2014; Kaloyianni *et al*., 2009; Matozzo *et al*., 2001). PAHs have been considered also modulator of bivalve immune system (Ellis *et al*., 2011; Giannapas *et al*., 2012; Grundy *et al*., 1996; Lowe and Pipe, 1994; Pipe *et al*., 1995). It has also been described that pollutants inhibit phagocytosis in hemocytes. They also inhibit lysosomal membrane stability (Grundy *et al*., 1996) or the increase of ROS production by hemocytes, considered an important mechanism used by bivalves for killing microorganisms and parasites (Dyrinda *et al*., 1997; Hegaret *et al*., 2003).

6.2. Physiological biomarkers

Various physiological parameters have been used as pollution biomarkers. Basic physiological functions as feeding, absorption or respiration can provide information about the presence of pollutants. Organisms are able to adapt their physiological mechanisms when they are subjected to an external stress. As a consequence, the physiological parameters can be considered as a useful tool for environmental assessment. Moreover, all of these vital functions (filtration, ingestion, absorption and respiration), can be integrated in the energy budget equation of Winberg (1956) from which the scope for growth (SFG) concept is derived. The determination of growth in organisms is one of the most sensitive methods available to detect, quantify and identify changes of the water quality of marine ecosystems over time and space. That is possible because, growth is the result of a combination of different physiological processes involved in energy acquisition and consumption. It is, therefore, a non-specific, highly sensitive, comprehensive and ecologically relevant response of organisms to the presence of a contaminant (Albentosa *et al*., 2012). SFG is a technique involving the calculation of the energy available for growth under standardized laboratory conditions (Widdows and staff, 2006). In summary, it consists on evaluating the energy acquired by an organism after absorbing the ingested food, subtracting the energy lost from respiratory and excretory processes. The presence of contaminants in the marine environment changes this energy balance, making SFG a suitable marker for toxic stress (Albentosa *et al*., 2012; Bellas *et al*., 2014; Halldörson *et al*., 2005; Widdows *et al*., 1995; Widdows *et al*., 2002). Therefore, SFG is a biomarker at the individual/whole organism level of biological complexity, with a high level of ecological relevance and very useful in monitoring programs (ICES, 2014).

A negative effect on SFG caused by organochlorine compounds has been described on SFG in field studies (Widdows *et al*., 1997; Wang *et al*., 2005), by PAHs under both laboratory conditions (Donkin *et al*., 1989; Eertman *et al*., 1995; Widdows and Donkin, 1991) and field studies (Widdows *et al*., 1995; 2002), and also by metals (Anderlini, 1992; Chandurvelan *et al*., 2012) or by a mix of pollutants (Campillo *et al*., 2013; Perpétua *et al*., 2015). In addition, not only SFG, but also the isolated physiological parameters that comprise the SFG, have been considered indicators of the effects caused by pollution exposure. For example, feeding inhibition has been observed in the presence of PAHs (Al-Subiai *et al*., 2012; Beiras *et al*., 2012; Toro *et al*., 2003) and metals (Sobrino-Figueroa and Cáceres-Martinez, 2014).

6.3. Biochemical biomarkers

Biochemical biomarkers are extensively used in environmental monitoring as part of integrated programs together with analyses of contaminants (Cajaraville *et al*., 2000; ICES 2007; Viarengo *et al*., 2007). As mentioned above, organisms exhibit several mechanisms at the cellular level to protect themselves from the toxic effects of organic and metallic compounds in polluted environments. This have promoted that some oxidative (SOD, CAT, GR, GPx) and detoxification markers (GST) have been extensively used to reveal the exposure to metals and organic compounds in the environment (e.g. Campillo *et al*., 2013; Fernández *et al*., 2010,2012; Regoli, 1998; Vidal-Liñán *et al*., 2010).

In this sense, it is possible to find several studies in which antioxidant responses have been used in mussels to assess the effect of pollution, including organic and metallic compounds. For instance, the alteration of the enzymatic responses of CAT (Eertman *et al*., 1995; Fernández *et al*., 2010; Porte *et al*., 1991; Sureda *et al*., 2011; Regoli and Principato, 1995;), SOD (Eertman *et al*., 1995; Pan *et al*., 2005; Richardson *et al*., 2008; Rocher *et al*., 2006; Cheung *et al*., 2001), GR (Box *et al*., 2007; Solé *et al*., 2007; Maria and Bebianno, 2011) or GPx (Cheung *et al*., 2001; Tsangaris *et al*., 2007; Sureda *et al*., 2011) have been described in presence of organic and metallic compounds. Although it is not an specific antioxidant enzyme, GST activity has been widely used in mussels as a biomarker of exposure to organic compounds (Fiztpatrick *et al*., 1997; Cheung *et al*., 2004; Rocher *et al*., 2006; Richardson *et al*., 2008), organochlorinated compounds (De Luca-Abbott *et al*., 2005) and metals (Borkovic *et al*., 2005; Lee *et al*., 1998; Funes *et al*., 2006).

However, contradictory results regarding the behaviour of enzymatic responses to pollution have been usually reported by those studies. This can be explained by the fact that the induction of CAT, GST or GPx activities frequently occurs at low intensity of exposure, showing a compensatory increase to overcome raised oxidative stress. However, with the increase of oxidative pressure, the initial induction can be followed by a progressive decrease of enzymatic activities, up to their severe depletion (Regoli and Giuliani, 2014; Viarengo *et al*., 2007).

On another hand, LPO has been considered as damage indicator for cellular membrane lipids caused by ROS and is useful to assess exposure to and the effects of pollutants in mussels (Campillo *et al*., 2013; Cheung *et al*., 2001; ICES, 2013; Regoli and Principato, 1995; Regoli, 1998).

7. What are "*confounding factors*" and how they affect biomarkers?

Biomarkers have been extensively used to assess the effect of chemical compounds in organisms, providing a first biological signal of exposure (Cajaraville *et al*., 2000; Lam, 2009; Van der Oost *et al*., 2003). However, despite the considerable knowledge of their links with contaminant exposure, biomarkers are not yet completely implemented in national and international monitoring programs (e.g. JAMP, HELCOM-BSAP, MEDPOL).

In the ICES Working Group on the Biological Effects of Contaminants (WGBEC) meeting celebrated in France (2008), it was pointed out that "*the **ideal biomarker** would only be modulated by contaminants and not by other variables such as natural physicochemical parameters; but, in reality, this is highly unlikely to occur*" (ICES, 2008). In fact, biomarkers data are often difficult to interpret due to the large number of natural variables affecting them. To avoid the misinterpretation of responses to pollution, studies about the environmental influence on different biomarkers could be an important contribution to the establishment of reference activity levels against which biomarker changes can be estimated (Menezes *et al*., 2006).

In this regard, it has been highlighted the existence of ***confounding factors*** which can alter biomarker responses to pollution (ICES, 2006; Coulaud *et al*., 2011; Lam, 2009). A ***confounder*** is conventionally defined as a variable that is correlated (or more generally, associated) with a stressor of interest and has a causal effect on the response of interest (Farrar *et al*., 2015). In our context, ***confounding factor*** can be defined as any endogenous or exogenous factor in the bioindicator that could be masking the biological responses to pollution. Figure 10 shows an schematic overview of how a confounding factor can interfere in the biomarker responses to pollution.

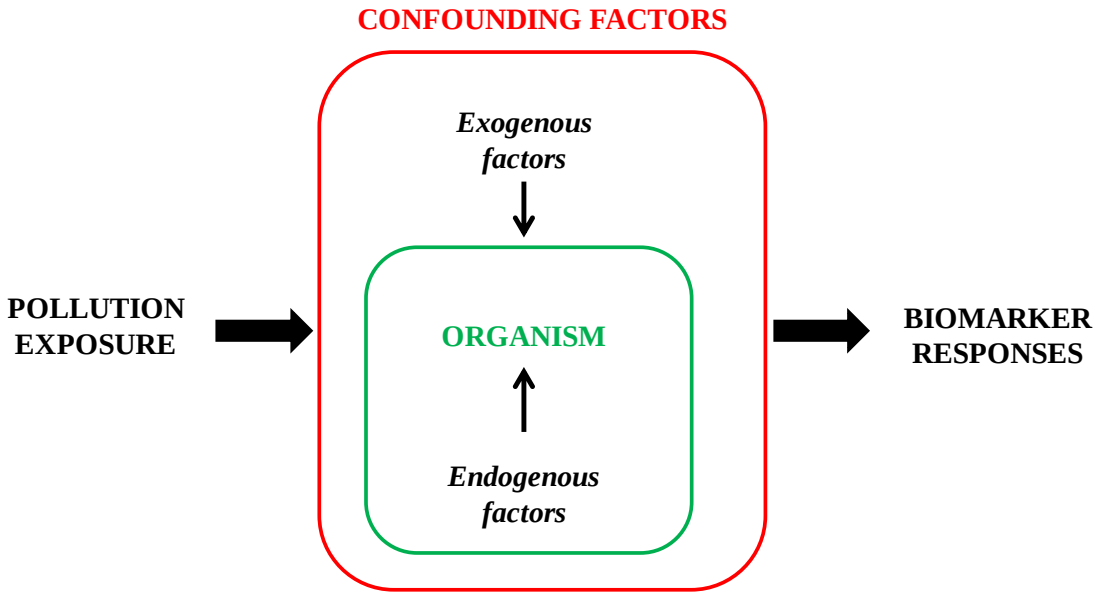


Figure 10. Schematic overview of mechanism of interference of confounding factor in the biomarker responses to pollution. Personal elaboration.

The concept of confounding factor was first considered in the WGBEC in 2006 as a factor that needed to be taken into account when designing of an integrative biological monitoring program and, in this case, was specifically required to assess data from a post spill monitoring program (ICES, 2006). This subject was deeply discussed during the 2008 meeting (ICES, 2008) in which it was suggested that the risk of chemical substances can be altered by environmental variables. Thus, one of the major problems limiting the application of biomarkers in the evaluation of marine pollution is related to their natural variability, since changes in these biological responses are not only related to chemical pollutants, but also to environmental factors (e.g. temperature, salinity, food availability or dissolved oxygen levels), as well as to intrinsic biological cycles (e.g. reproductive cycle) (Widdows, 1978).

In the context of monitoring programs, and foremost, monitoring programs using mussels as bioindicators of pollution, some exogenous factors such as temperature (Viarengo *et al*., 1998), differences in air exposure during low tide (Schmidt *et al*. 2012; Vidal-Liñán and Bellas, 2013), salinity variations (Prevodnik *et al*., 2007), food availability (Martínez-Álvarez, 2005) or endogenous factor such as reproductive cycle (Borkovic *et al*., 2005; Sheehan and Power, 1999), or mussel age (Zilberberg *et al*., 2011) have been suggested to alter biomarker responses to pollution.

Temperature has been considered as one of the most important factor in the physiology of organisms (Zippay and Helmuth, 2012). Temperature can affect organisms responses to pollution in several ways. Fistly, it can cause physiological changes by itself but it can also alter the availability of pollutants in the marine environment, increasing their bioavailability due to changes in the kinetic reactions of chemicals (Leon *et al*., 2004). Several authors have evidenced the effect caused by an increase of temperature in mussel biomarker responses (Viarengo *et al*., 1998; Jarque *et al*., 2014; Yao and Somero, 2013).

Contrary to other organisms, mussels do not have a size limit during the growing process. In fact, it is very characteristic of bivalves to have a longitudinal growth which is prolonged along their life cycle (Sukhotin *et al*., 2002). The shell grows gradually, forming calcareous rings which could be identified as years, months or even days. For this reason the shell's growth may be used as indicative of mussel's ***age*** (Frew *et al*., 1989). Thus, *shell thickness* has been shown to give a good indication of the relative age of bivalves (Frew *et al*., 1989; Yap *et al*., 2003). However, the growth of mussels is mainly determined by the environmental conditions. As a consequence, when mussels are not in optimum conditions of food and temperature, their growth pattern may be altered and the shell becomes thicker. In this sense, previous studies have pointed out the effect of the mussel's age on biomarker parameters (Sukhotin and Pörtner, 2001, Sukhotin *et al*., 2002; Zilberberg *et al*., 2011). Mussels used in monitoring programs are selected by size in order to eliminate the "age" factor but, sometimes, and as a consequence of the great variability of environmental conditions between sampling

sites, mussels of the same size would have different age, which would acts as a confounding factor.

Previous studies carried out within our research group (Albentosa *et al*., 2012; Bellas *et al*., 2014) have revealed that physiological and biochemical biomarkers seems to be more affected by ***mussel condition*** (measured as condition index) than by chemical pollution. This challenge is especially critical in large-scale monitoring programs where large geographical variability in environmental characteristics is observed. Mussel condition is commonly assessed by measuring the condition index (CI) which, among others, is considered as the ratio of flesh to shell dry weights (Lucas and Beninger, 1985). It is well known that CI is determined by external factors as food availability or temperature, and internal factors as gametogenesis (Lucas and Beninger, 1985; Filgueira *et al*., 2013), which interact in marine ecosystems.

Food availability has been widely considered as one of the most variable factors in the marine environment, especially in the intertidal habitats where mussels live. Large scale-monitoring programs are characterized by a great variability of food conditions between sampling sites, seasons and sampling years, which can promote differences in mussel's biological processes. The effect of food availability on biomarker responses has been poorly studied. Most of the studies regarding food conditions and biomarker responses are related to starvation (no food) in fish (Hidalgo *et al*., 2002; Pascual *et al*., 2003; Morales *et al*., 2004) or crabs (Matozzo *et al*., 2011). In mussels, most of the studies are focussed on how natural variability of environmental conditions induces differences in biomarkers responses. Seasonal variations in physiological responses such as clearance rates and respiration rates have been evidenced in mussels (Crandford *et al*., 2011 and references therein; Fernández *et al*., 2010; Irisarri *et al*., 2014). Moreover, several authors have described the seasonal variability of antioxidant enzymes as CAT or GPx (Livingstone, 2001; Botichelli and Regoli, 2006; Vidal-Liñán *et al*., 2010) and detoxification enzymes such as GST (Leiniö and Lehtonen, 2005), related to the influence of the food availability. A recent study carried out in natural field conditions (Dowd *et al*., 2013) concluded that it is the food availability, more than body temperature, the factor that has a strongest correlation with ATP-generation and antioxidant enzyme capacities in a population of intertidal mussels, *M. californianus*. Moreover, mussels are opportunistic species (Bayne, 1976), thus, if environmental conditions (food availability and high temperature) are favorable, they develop their gonadas. As a consequence, the reproductive cycle of mussels and is intimately linked to the food availability conditions (Gabbott, 1975) and it is very difficult to separate both factors to study the response of biomarkers to pollution under natural conditions.

Large variations in food supply are not only related to food availability, but also to species composition of the phytoplankton community (***food quality***). Phytoplankton communities vary both temporally and spatially in response to physical and biological

factors (Navarro and Thompson, 1993). The size and composition of phytoplankton depends on the concentration of nutrients in the water column, zooplankton grazing and hydrodynamics (Cermeño *et al*., 2006). Among the different phytoplankton groups, diatoms and dinoflagellates are known to prevail under different oceanographic conditions (Oliveira *et al*., 2009). In this sense, natural (upwelling) and anthropogenic eutrophication processes modify phytoplankton patterns depending on their specific nutrient requirements. In general, diatom blooms dominate during the spring and summer upwelling fertilization events, whereas dinoflagellate blooms are more abundant in nutrient poorer waters or during stratified conditions in summer (Oliveira *et al*., 2009; Smayda and Trainer, 2010). In addition, anthropogenic eutrophication that is characteristic of semi enclosed coastal ecosystems, also causes a modification of nutrients composition which affects species composition of phytoplankton communities. Excessive N-inputs in eutrophic systems cause a decrease in Si:N ratios which leads to a proliferation of non-siliceous species as dinoflagellates in contrast to diatom dominance (Fouillaron *et al*., 2007). Differences among phytoplankton species are related to cell size, toxicity, cell wall composition, proximal composition or essential nutrients such as fatty acids, which influence energy and nutrient uptake by filter feeders. Biological responses used as pollution biomarkers can be influenced by these particular food properties (food quality), as it was above pointed out with food availability (food quantity). However, it remains unexplored how changes in primary production quality could affect the biomarker responses upon exposure to pollutants in large-scale monitoring programs where a wide range of phytoplankton communities can be found at the same time.

Reproductive cycle is another factor that has a high influence on the mussel condition and needs to be also considered. Mussels evidence an annual cycle in which it is possible to differentiate a resting period, a mature period and a spawning period as it was previously described (*section: 5.4*). Gonadal development is an energy demanding process which requires high food availability conditions or body reserves (Gabbott, 1975). In large-scale programs, variability of natural trophic conditions between sampling sites, leads to differences in mussel gonadal development and the whole range of gametogenic stages may coexist at the same sampling period, even when sampling is carried out during the theoretically resting stage for the species considered. This is especially true for opportunistic species such as the common mussel, which, if environmental conditions (food availability and high temperature) are favorable, builds up an important gonadal development (Bayne, 1976). For that reason, in mid-latitudes, as is the case of the Spanish coast, the cyclical nature of gametogenesis in *Mytilus* is less evident. Although the reproductive investment could directly affect biomarker responses to pollution, studies on mussels which consider the reproductive cycle as a key factor on pollution biomarker responses under controlled laboratory conditions are hardly found in literature. The closest literature on this subject is referred to field studies about the seasonal variability of biomarkers' responses in native (Bocchetti and Regoli, 2006; Borković *et al*., 2005; Faria *et al*., 2014; Hagger *et al*., 2010; Nahrgang *et al*., 2013; Power and Sheehan, 1996; Schmidt *et al*., 2013; Sheehan and Power, 1999;

Vidal-Liñán *et al*., 2010) or transplanted mussels (Bodin *et al*., 2004; Giarratano *et al*., 2011; Palais *et al*., 2012). However, in these seasonal field studies, it results impossible to discern which process, gametogenesis or nutrition, is responsible of animal condition and which of them is influencing the biomarker responses.

In conclusion, the availability of solid assessment criteria for biological effect methods to be included in national or international monitoring programs is a requirement of international environmental agencies (e.g. ICES, 2014). The development of assessment criteria demands an extensive knowledge of the influence of natural conditions on biomarker responses (Tankoua *et al*., 2011). The high complexity of marine ecosystems where several variables, as nutritive and reproductive conditions interact, difficults the comprehension of biomarkers responses to pollution. The successful implementation of biomarkers in environmental monitoring programs requires a good understanding of the mechanisms underlying these responses (Van der Oost, 2003). Thus, laboratory experiments, as those presented in this thesis, where variables are isolated, modified and controlled, are crucial to assess the biological effects of pollutants under different natural environmental scenarios.

HYPOTHESIS AND OBJECTIVES

HYPOTHESIS AND OBJECTIVES

1. Hypothesis

The general hypothesis of this thesis is that large-scale monitoring programs are affected by ***mussel condition*** (nutritive and reproductive condition), which mask the responses of the biomarkers to pollution.

2. Objectives

According to the hypothesis of this thesis, the main objective was to identify how mussel condition is affecting biomarker responses to pollution. For this purpose, the following specific objectives were proposed:

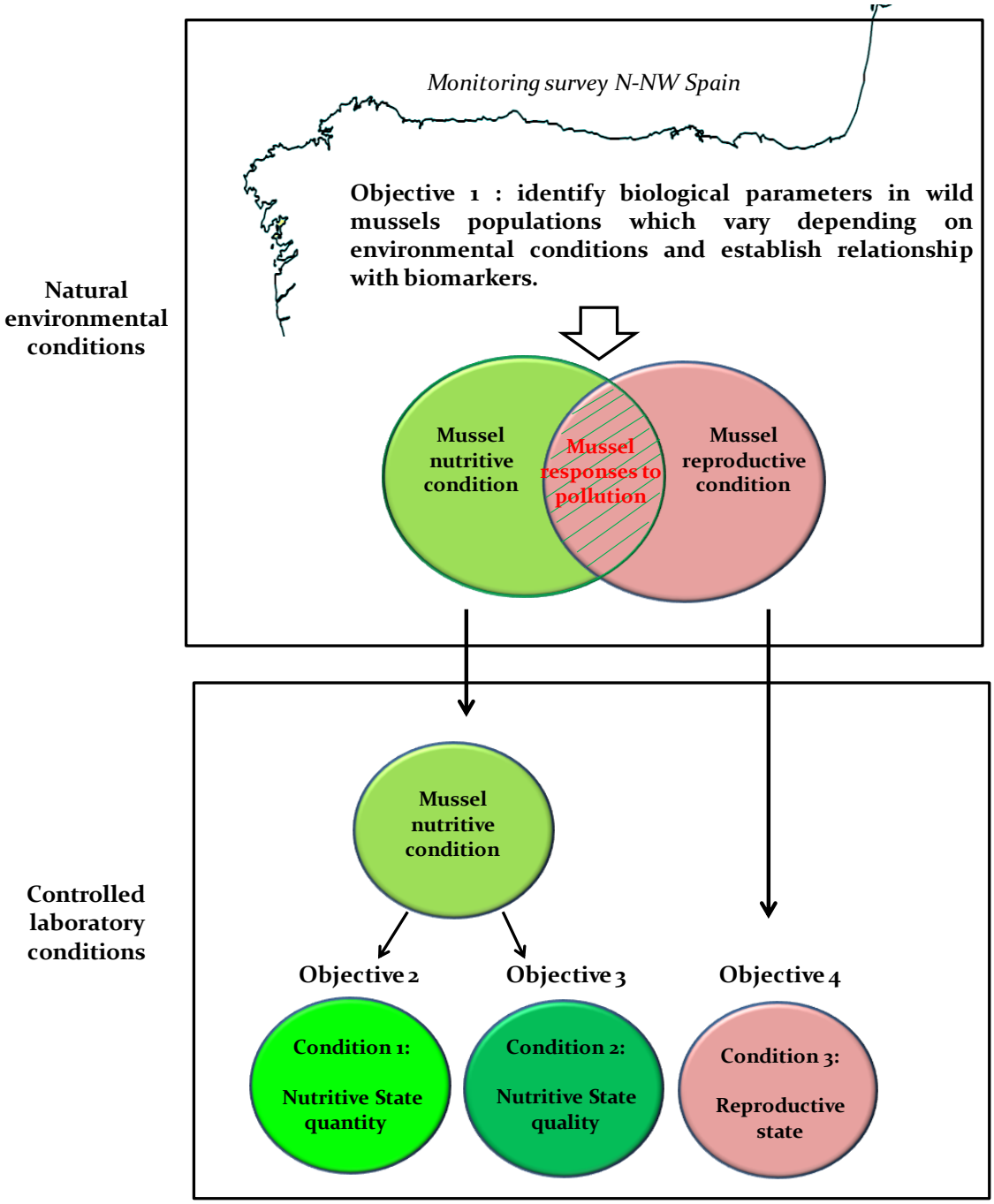
1. To assess the main biological variables that could be acting as confounding factors in monitoring programs by establishing relationships between i) biological variables and biomarkers, and ii) biomarkers and pollution, using wild mussel populations. Results of this objective are exposed in **Chapter 1** of this thesis.

2. To study the effect that ***mussel nutritive state (condition I: quantity)*** has on biomarker responses to pollution under controlled laboratory conditions. Results are shown in **Chapter 2**.

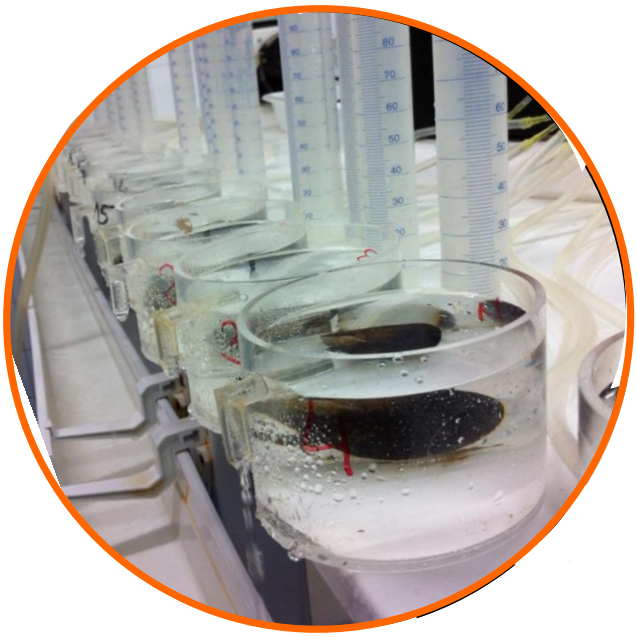
3. To study the effect that ***mussel nutritive state (condition II: quality)*** has on biomarker responses to pollution under controlled laboratory conditions. Results are presented in **Chapter 3**.

4. To assess the effect of the ***mussel reproductive state (condition III)*** on mussel biomarker responses to pollution under controlled laboratory conditions. Results are discussed in **Chapter 4**.

THESIS EXPERIMENTAL DESIGN



MATERIAL AND METHODS



Carmen González Fernández, 2016

MATERIAL AND METHODS

1. Field study

1.1. Mussel collection

Mussels (40-60 mm in length), *Mytilus galloprovincialis*, were collected from 23 sites along the N-NW Spanish Atlantic coast, in November 2012, at low tide and transported in cold and air exposed, to the IEO's laboratories. These sampling sites were chosen among those considered within the spatial distribution studies of the Spanish Marine Pollution Program (SMP) (Figure 11). The SMP is carried out since 1990 within the framework of the JAMP (OSPAR Convention). Sites cover a wide range of environmental scenarios (pollution sources, industrial areas, cities, upwelling, etc.).

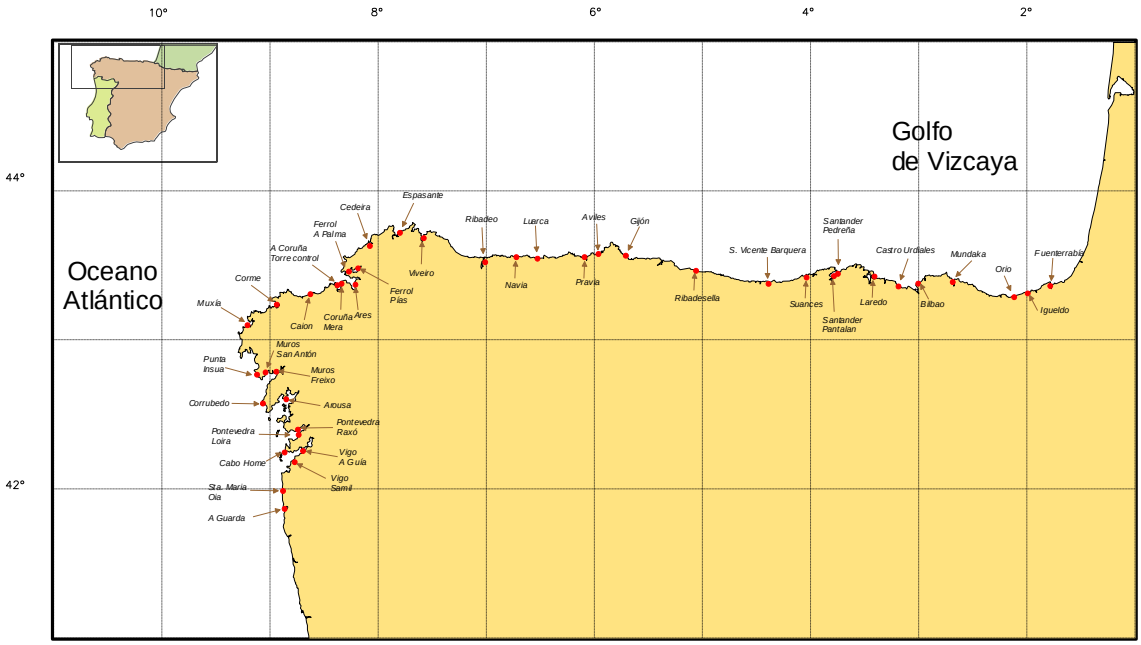


Figure 11. Geographical situation of sampling sites included in the Spanish Marine Pollution Monitoring Program located in the N-NW Spanish coast.

1.2. Environmental scenario

The Atlantic survey of the SMP is located in the OSPAR Region IV and covers more than 2500 km of the N-NW Spanish coastline. This survey is usually carried out in autumn (November), when mussels are in a more stable physiological state, according to the JAMP Guidelines for Monitoring Pollutants in Biota (OSPAR Commission, 2010) and consist of 40 sampling sites, from which 23 sites are monitored yearly. The sampling area includes two different oceanographic regions: the Atlantic and the Cantabrian coasts in the Bay of Biscay, which differ in their trophic and hydrodynamic characteristics. The wind intensity and strength on the Galician platform provides upwelling processes mainly at the end of the spring, with maximum values in summer.

The intensity of upwelling decrease from Cabo Finisterre to Santander, which is considered the limit of the Iberian upwelling (Lavín *et al*., 2012; Molina, 1972). The Atlantic coast has also shown several periods of upwelling in late summer or autumn (Bode *et al*., 1996, 2011; Botas *et al*., 1990). The Cantabrian coast shows the upwelling phenomenon between the late spring and summer but with less intensity and duration than in the Atlantic coast (Gil, 2008; Lavín *et al*., 1998; Llope *et al*., 2003). In summary, Atlantic waters are more productive than those of the Cantabrian zone, due to the greater abundance and intensity of upwellings processes from which nutrient-rich water rises from bottom fertilizing surface waters (Cermeño *et al*., 2006; Fraga, 1981). Table 2 shows the location of sampling sites and their environmental characteristics. The trophic characteristics were established according to Ospina-Alvarez *et al*. (2014) which using the primary production as an indicator, establish the limit of the highly Atlantic productive rías in Estaca-de-Bares from where primary production and upwelling event intensity decrease.

Table 2. Location and environmental characteristics of the 23 sampling sites along the N-NW Spanish coast. Numbers under Environmental Conditions relate to the degree of waves exposure (1:protected; 2:less protected; 3:Moderately exposed; 4: exposed and 5:heavily exposed) and letters are related to trophic conditions (H: hypertrophic and O: oligotrophic conditions) according to Ospina-Alvarez *et al.* (2014).

Sampling site	Code	Latitude	Longitude	Environmental conditions
		Atlantic coast		
Oia	2	41º58.212	08º53.199	5 H
Samil	3	42º13.177	08º46.604	2 H
Cabo Home	5	42º15.007	08º52.333	5 H
Pontevedra Raxó	7	42º24.172	08º44.968	2 H
Chazo	8	42º36.374	08º51.761	2 H
Punta Insua	12	42º46.423	09º07.550	5 H
Corme	14	43º14.569	08º56.642	3 H
A Coruña TC	16	43º22.178	08º23.160	5 H
Ferrol Palma	19	43º27.770	08º16.191	1 H
Cedeira	21	43º38.638	08º05.024	2 H
Espasante	22	43º43.346	07º48.302	4 H
		Cantabrian coast		
Ribadeo	24	43º31.570	07º01.032	1 O
Luarca	26	43º32.872	06º32.409	3 O
Avilés	28	43º34.759	05º58.180	4 O
Gijón	29	43º34.174	05º43.329	5 O
Ribadesella	30	43º28.051	05º03.742	2 O
SV Barquera	31	43º22.885	04º23.803	1 O
Suances	32	43º26.338	04º02.621	3 O
Santander Pantalán	33	43º25.937	03º47.476	1 O
Santander Pedreña	34	43º26.929	03º45.173	1 O
Castro Urdiales	36	43º21.868	03º11.663	3 O
Bilbao Azcorri	37	43º22.917	03º00.885	5 O
Hondarribia	41	43º22.119	01º47.474	1 O

1.3. Samples preparation

A total of 150 mussels were used for chemical analysis (3 pools of 50 mussels per group) where soft tissues were separated from the shells sand triturated with Ultra-turrax. Another 30 mussels were acclimated to laboratory conditions, placed in filtered seawater (1 µm) under controlled temperature (15 ºC) and feeding conditions (microalgae *Isochrysis galban*a, clone T-ISO, at a ratio of 8 % of microalgal organic matter per soft tissue dry weight) in an aerated close system to recover from the

prolonged air exposure and transportation. Within this group, 15 mussels were used for physiological analyses. Subsequently to physiological measurements (clearance, absorption, respiration and scope for growth), mussels were dissected and valves, palps, gills, mantle, glands and remaining soft tissues were weighted for biometry analyses. Mantles of mussels (n=15) were used for histology whereas gills and digestive glands were conserved in liquid nitrogen (-80 ºC) for biochemical biomarker analyses. Biochemical biomarker analyses were performed in mussel´s digestive glands making pools of 2 individuals (n=5 pools).

2. Laboratory studies

2.1. Contaminant model: Fluoranthene (FLU)

Fluoranthene (FLU) was selected as model polycyclic aromatic hydrocarbon (PAH) because it is included in the lists of priority substances in the field of water policy of the European Commission (EC) and the United States Environmental Protection Agency (USEPA). FLU is considered to be the most toxic PAHs for marine biota in the short term (Ben Othman *et al*., 2012) and it is one of the PAHs found in higher concentrations in sediments, particulate matter and water (Baumard *et al*., 1998; van Hattum *et al*., 1998; Viñas *et al*., 2010). It chemical structure is shown in Figure 12. It is a molecule consist of three aromatic rings and has a higher lipophilic character than other compounds of the same group, consequently, being one of the most abundant PAHs accumulated in molluscs (Baumard *et al*., 1998; Bouzas *et al*., 2011; Bellas *et al*., 2014).

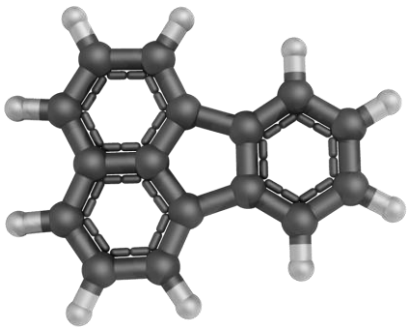


Figure 12. Fluoranthene chemical structure.

2.2. Condition I: Nutritive State-Quantity

2.2.1 Mussel collection

Wild mussels (4.5 cm), *Mytilus galloprovincialis*, were collected from site 02 (Santa María de Oia) of the SMP, an unpolluted site from Galicia (NW Spain), and transported in cold and air exposed, via overnight express delivery services (less than 24 h) to the IEO's laboratories (Murcia) where the experiment was carried out. Mussels were acclimated to laboratory conditions (filtered seawater 0.5 µm, 15 °C) in an aerated closed system.

2.2.2 Conditioning period: production of different quantitative nutritive conditions

Mussels were fed with 3 different rations of the microalgae *I. galbana*, clone t-ISO, for a period of 56 days in order to create a range of mussel energetic conditions according to previous results published in Albentosa *et al*. (2012b)(Figure 13). Rations were 0.05 (NS-1), 0.15 (NS-2) and 0.35 (NS-3) % of microalgal organic matter (OM) per mussel live weight. Mussel ingestion (I, energy inputs) was recorded daily and mussel respiration (R, energy outputs) was measured at the beginning and at the end of the conditioning period. Mussel growth (G) during conditioning was also recorded. Measurements were integrated for the whole condition-ing period and used for the estimation of the energy lost in the feces (F) as follows: $F = I - R - G$. The difference between I and F was considered the energy absorbed $Ab = I - F$, from which the absorption efficiency (AE) could be estimated: $AE = Ab\ I^{-1}$.

Variations in the mussel biochemical composition were registered during the conditioning period and used to estimate the difference in mussels' energy balance before and after the conditioning period. This balance was considered as an indicator of mussel metabolism. Biochemical composition was standardized to the total organic content of the samples, considering that the non-quantified organic matter has the same composition than that quantified. Biochemical contents were transformed to energetic values using a conversion factor of 35.2 J mg OM^{-1} for lipids, 17.2 J mg OM^{-1} for carbohydrates and 18 J mg OM^{-1} for proteins (Whyte *et al*., 1990).

Figure 13. Mussel conditioning to three algae rations (left) and one mussel condition (right) carried out under controlled laboratory conditions. NS-1: Low ration, NS-2: Intermediate ration and NS-3: High ration.

2.2.3 Exposure

Mussels were exposed to the polycyclic aromatic hydrocarbon (PAH) fluoranthene (FLU) at three conditions: Control, Low and High exposure. Experiment was designed in such a manner that FLU exposure, expressed as both, total µg FLU offered per g of mussel dry tissues (µg FLU g dw^{-1}) and FLU concentration (µg FLU L^{-1}), were the same for the three mussels conditions. This experimental premise was achieved by adjusting tank volume, due to the important differences in individual

mussel weights, obtained with the conditioning. The exposure experiment was carried out in 9 tanks (3 mussels conditions, NS-1, NS-2, NS-3 and 3 FLU exposures Control, Low, High) with 35 mussels each one. Low FLU exposure was tested at a concentration of 3 µg FLU $L^{-1}$and a dose of 125 µg FLU g dw^{-1}. High FLU exposure condition was supplied at a concentration of 60 µg FLU $L^{-1}$and a dose of 2500 µg FLU g dw^{-1}. FLU stock solutions were prepared in acetone for each tested concentration. Control treatments included only the acetone solvent at a concentration of 30 µg L^{-1}. Exposure conditions were maintained for a period of 3 weeks.

Mussels were fed (*I. galbana*, clone T-ISO) during exposure at the same rations as used in the conditioning period. During exposure, tanks were covered in order to reduce toxicant volatilization and degradation. Experimental solutions were renewed every 24 h to maintain water quality and FLU concentrations. Moreover, FLU stock per tank was mixed daily with the amount of microalgae giving an initial food concentration in the tanks of 2 mm^3 L^{-1}. Daily FLU doses were incubated with this fraction of microalgae, in agitation with light for 30 minutes (Figure 14). Then, the toxicant was distributed within each tank. An ingestion test was carried out daily, after each FLU addition, in order to check the toxicity of FLU. To do this, microalgae concentrations (nominal concentration for the test 2 mm^3 L^{-1}) were measured using a Coulter Counter Multisizer III just after FLU addition and 15 min later.

Figure 14. Mussel exposure carried out under controlled laboratory conditions (left) and preparation of toxic (food+toxicant) (right).

2.2.4 Samples preparation

After exposure, 30 mussels were collected from each condition treatment. 15 mussels were used for physiological analyses (clearance, absorption, respiration and scope for growth), , After that, mussels were dissected and valves, palps, gills, mantle, glands and remaining soft tissues were weighted in order to determine biometric parameters. In this experiment, mussel tissues were pooled (3 pools, 5 mussels each) and triturated with Ultra-turrax in order to determine mussel biochemical composition (reserves) and chemical (fluoranthene) accumulation. Other 15 mussels from each

condition treatment were selected and gills, digestive gland and mantle tissues were dissected. Mantle tissues were used for histology (n=15) whereas 10 mussels were used for biomarker analyses making pools of 2 individuals (n=5). Biochemical Biomarkers analyses were performed in mussel´s digestive glands.

2.3. Condition II: Nutritive State-Quality

2.3.1. Mussels collection

Mussels (*Mytilus spp.)* from the Armorique cape in the Bay of Brest (48°19'20.29"N, Brittany, France), were collected at low tide in September - October 2013, to minimize possible physiological differences between animals. Mussels were transported to LEMAR´s laboratories where the experiment was carried out. Once in the laboratory, mussels were distributed in two 100 l tanks to acclimate them to standardized conditions (1 µm-filtered seawater, 17 °C, aerated open flow system).

2.3.2 Conditioning period: production of different qualitative nutritive conditions

Two different diets were supplied at 3% of microalgal organic matter per soft mussel´s tissue dry weight for 6 weeks in order to create 2 different types of mussel nutritive conditions: C-mussels (mussels fed with *Chaetoceros neogracile*) and H-mussels (fed with *Heterocapsa triquetra*) (Figure 15). Algae concentration was quantified daily with a flow cytometer before supplying them to the mussels. Furthermore, algal quality and fatty acid profile were determined weekly in order to regulate algae physiological state.

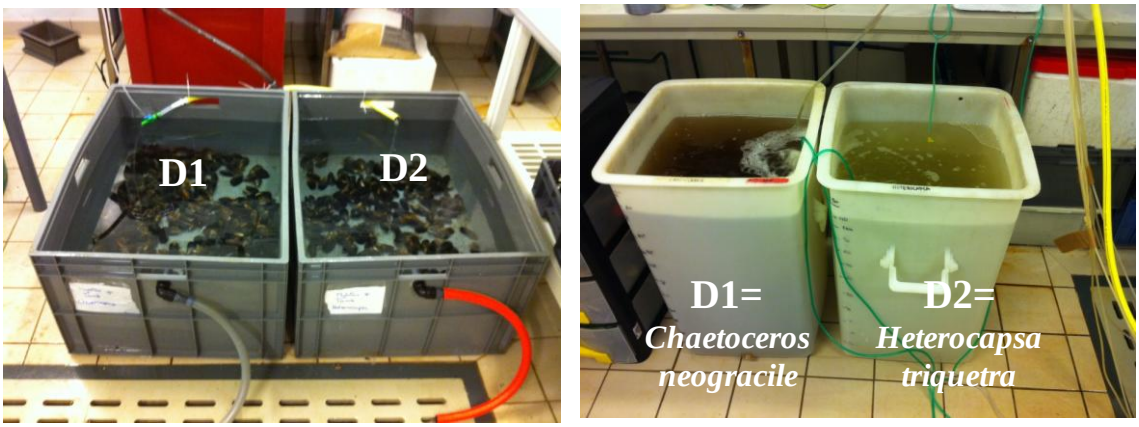


Figure 15. Mussel conditioning to two algae species (left) carried out under controlled laboratory conditions and conditioning algaes (right). D1: *Chaetoceros neogracile* and D2: *Heterocapsa triquetra*

2.3.3. Exposure

After conditioning, between 25 - 28 mussels were distributed in twelve 40 L tanks. Mussels were exposed to FLU for 1 week to a concentration of 30 µg L^{-1} FLU per day and an equivalent dose of 390 µg FLU g dw^{-1}. The daily FLU dose was mixed with algae (each type of algae separately) for 45 min in the dark, before feeding the mussels. Microalgae were supplied with a peristaltic pump giving 1.5 % of microalgal

organic matter per soft tissue dry weight (*C* or *H*) in an aerated close system (Figure 16). Control treatments only carried the solvent (acetone). Tanks were kept in dark in order to avoid degradation of FLU. Experimental solutions were totally renewed every 24h

Figure 16. Mussel exposure carried out under controlled laboratory conditions. Mussels tanks (left) and food+toxicant supplied during the exposure through a peristaltic pump (right).

2.3.4. Samples preparation

After exposure, 22 mussels were collected per condition. Hemolymph of 12 mussels was extracted for immune analyses (hemocyte´s viability, phagocytosis and hemocyte´s ROS production) and, subsequently, mussel´s digestive glands were dissected in RNase-free conditions and quickly snap-frozen in liquid nitrogen and stored individually at -80°C. Organs were subsequently ground into powder under liquid nitrogen using a mixer mill MM400 (Retsch) to obtain a homogenized powder and divided for further chemical, biomarker and fatty acid (FA) analyses. Three mussels from each condition were used for FA analyses. The remaining 9 mussel were used for individual chemical quantification (FLU accumulation) and individual biochemical biomarkers analyses using a sonicator UP 200S (0.5 cycle and 60% of amplitude with two rounds of 5 pulses) to homogenize tissues. The other 10 mussels were used to determine the biometric parameters. Valves, palps, gills, mantle, glands and remaining soft tissues were weighted for biometry and then, mussel tissues were pooled (3 pools, five mussels each) and triturated with Ultra-turrax in order to determine mussel biochemical composition (reserves) and total chemical (FLU) accumulation.

2.4. Condition III: Reproductive State

2.4.1. Mussel collection

Two sampling seasons were considered in this study in order to cover the mussel reproductive cycle. Wild mussels, *M. galloprovincialis*, were collected from the same site as described for Condition I experiment: site 02 (Santa María de Oia) of the SMP twice, in February (when mussels are expected to be in a Reproductive Stage, RS-1) and September (when mussels are expected to be in a Resting Stage, RS-2) 2013. After collection, mussels were transported in cold and air exposed, via overnight express

delivery services (less than 24 h) to the IEO's laboratories (Murcia) where the experiment was carried out. Mussels were acclimated to standardized laboratory conditions for a period of 1 week (filtered seawater 0.5 µm, 15 °C) in an aerated closed system.

Moreover, after acclimation, at each sampling period, 15 mussels were collected (initial time) in order to determine biological and histological characterization prior exposure with the objective of i) confirm that mussels were at the reproductive stage desired and ii) determine mussel weight, in order to expose mussels to the same toxicant dose in the two reproductive stages studied.

2.4.2. Exposure

After acclimatization, mussels were exposed to FLU for 3 weeks at three conditions: control, low FLU exposure (Low) and high FLU exposure (High) exposure. Low was tested at a concentration of 3 µg FLU L^{-1} corresponding to a dose of 125 µg FLU g dw^{-1}. High was dosed at a concentration of 60 µg FLU L^{-1} corresponding to a dose of 2500 µg FLU g dw^{-1}. FLU stock solutions were prepared in acetone. FLU concentration was adjusted to the initial mussel dry weight in order to supply exactly the same FLU dose to mussels at the two reproductive seasons.

Exposure was carried out in 30 L tanks with 45 mussels each and considering 3 treatments: Control, Low and High, for each experimental time (Figure 17). Controls treatments included only the acetone solvent. Mussels were kept at the same conditions as acclimatization during exposure (fed ration 0.17 % *I. galbana*, clone T-ISO and 15 ºC of temperature). Tanks were covered during exposure in order to avoid toxicant volatilization and degradation. Water was totally renewed every 24h and re-dosed with appropriate quantities of FLU stock solutions. The FLU dose was mixed daily with the amount of microalgae required to obtain an initial food concentration of 2 mm^3 L^{-1}. FLU was incubated with the microalgae, in agitation with light, for 45 minutes. After that, the algae-toxicant mixture was distributed within each tank. The rest of the daily food ration (0.17%) was evenly distributed throughout the day by peristaltic pumps.

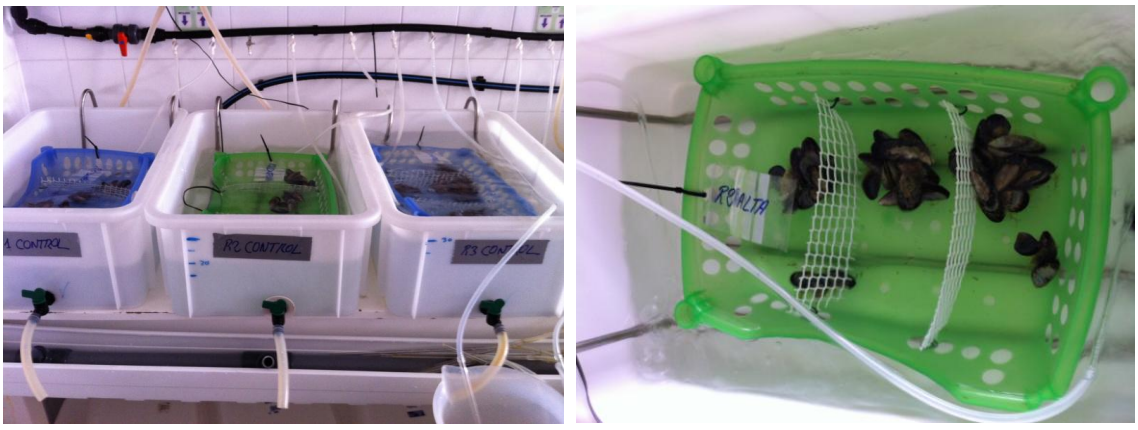


Figure 17. Mussel exposure to fluoranthene carried out at reproductive stage under controlled laboratory conditions.

2.4.3. Samples preparation

After exposure, 30 mussels were collected from each condition. Fifteenmussels were used for physiological analyses (clearance, absorption, respiration and scope for growth), and then were dissected and valves, palps, gills, mantle, glands and remaining soft tissues were weighted for biometric measurements. Mussel´s tissues were pooled (3 pools, 5 mussels each) and triturated with Ultra-turrax in order to determine mussel biochemical composition (reserves) and chemical (FLU) accumulation. Other fifteen mussels for each condition were selected and gills, digestive gland and mantle tissues were dissected. Mantle tissues were used for histology (n=15) whereas 10 mussels were use for biochemical analyses making pools of two individuals (n=5 pools). Biochemical biomarker analyses were performed in mussel´s digestive glands.

3. Biological measurements

3.1 Mussel biometry

Shell morphology was defined through the relationship between the three shell dimensions: (L/H), (L/W) and (H/W) (Alunno-Bruscia *et al*., 2001): length (L, maximum measure along the anterior-posterior axis), height (H, maximum dorsoventral axis) and width (W, maximum lateral axis), were measured to the nearest 0.1 mm with a calliper. Shell thickness (ST), considered as the average shell mass per unit area, has been shown to give a good indication of the relative age of bivalves (Frew *et al*., 1989; Yap *et al*., 2003). Shell surface area was gravimetrically measured by covering shells with aluminium foils, whose weights were directly related to the weight of a rectangular aluminium foil of known area (Griffin *et al*., 1980).

Subsequently, shells of mussels were carefully opened and valves, gills, mantle, glands and remaining soft tissues were weighed and dried at 100 ºC for 24 h to obtain individual dry tissue mass. The following biological indices were calculated:

GI (Gill Index) = (gills dry weight (total meat dry weight) $^{-1}$) × 100

GSI (Gonadosomatic Index) = (mantle dry weight (total meat dry weight) $^{-1}$) × 100

HI (Hepatosomatic Index) = (digestive gland weight (total meat dry weight) $^{-1}$) × 100

General mussel condition was evaluated through two substantially different condition indices (CI) according to Crosby and Gale (1990):

CI_{shell} (Shell Condition Index) = (total meat dry weight (shell dry weight) $^{-1}$) × 100

CI_{vol} (Volumetric Condition Index) = (total meat dry weight (mussel volume) $^{-1}$) × 100

Mussel volume, expressed in ml, and considered as related to internal mussel cavity, was calculated from shell dimensions as follows= $4/3 \times \pi \times (L/2) \times (H/2) \times (W/2)$.

3.2. Mussel gross-biochemical composition

Mussel biochemical components were quantified in three pooled samples of five mussels each one. Mussels' tissues were freeze-dried, ground with a mortar and pestle by hand. Protein content was determined following the method of Lowry *et al*. (1951), using bovine serum albumin as the standard. Carbohydrates were extracted by boiling the samples with 5% TCA and determined following Dubois *et al*. (1956) using oyster glycogen as the standard. Extraction of total lipids was carried out with mixtures of chloroform-methanol-water according to Bligh and Dyer (1959). Lipid content was established following Marsh and Weinstein (1966) using tripalmitin as the standard. Biochemical components were expressed as mg per g of ash-free dry weight (AFDW) soft tissues (mg g^{-1} AFDW). Carbohydrates were also expressed in absolute values for a mussel of a standard size of 8 ml (mussel volume) in order to avoid confusion due to relative percentages.

3.3 Fatty acid composition

Main specific differences between microalgae used in Condition II experiment were related to their fatty acid composition. Thus, in this experiment FA analyses were performed on both, microalgae (n=6; one per week of conditioning) and on mussel´s digestive glands (n=3, exposure time) after conditioning and exposure.

3.3.1. Lipid extraction of microalgae.

Three hundred milliliters of *C.neogracile* and *H.triquetra* cultures (about 2×10^6 cell mL^{-1} and 1×10^5 cell mL^{-1}, respectively) were sampled each week and filtered on a GF/F filters. At the end of the filtration, an additional volume of boiling distilled water (50 mL) was added and filtered to prevent lipid degradation through lipase activity. Total lipid extraction was conducted placing immediately microalgae filters in vials containing 6 mL of a chloroform/methanol mixture (2/1; v/v). To ensure a complete lipid extraction, vials were vortexed (3 times, 1 min) and sonicated (10 min, 4 ◦C). Total lipid extracts were then stored at −20 ◦C under nitrogen before analysis. The saturated fatty acid 23:0 (10 µL) was added to algae total lipid extract as an internal standard for FA quantitative measurements.

3.3.2. Lipid extraction and separation of mussel digestive glands.

Around 200 mg wet tissue were individually grounded and put in a tube containing 6 mL of chloroform–methanol mixture (2:1, v:v) To ensure a complete lipid extraction, vials were vortexed (3 times, 1 min) and sonicated (10 min, 4 ◦C). Total lipid extracts were then stored at −20 ◦C under nitrogen before analysis.

Neutral (NL) and polar lipids (PL) were separated on a silica-gel microcolumn (40 mm × 5 mm; Kieselgel Merck, 70–230 µm mesh) previously heated to 450 °C, deactivated with 6 % water as described by Soudant *et al*. (1995), and analyzed following the method described by Marty *et al*. (1992). Briefly, an aliquot of the sample (chloroform-methanol mixture) was evaporated to dryness, and lipids were recovered

with three washings of 500 µL each of chloroform-methanol mixture (98:2, v/v). Lipids were placed on top of the silica-gel microcolumn and neutral and polar lipids were eluted with 10 mL $CHCl_3$–MeOH (98:2, v/v) and 20 mL MeOH, respectively. A known amount of 23:0 fatty acid, as internal standard, was added in both neutral and polar fractions.

3.3.3. Esterification and fatty acid analysis.

Total lipids of algae and NL and PL of digestive glands were evaporated under nitrogen and transesterified with 800 µL of MeOH-BF_3–(10 %, v/v) for 10 min at 100 °C. After cooling, 800 µL of hexane saturated with distilled water and 1.5 mL of distilled water were added to each sample vial, then agitated and centrifuged. The aqueous lower phase was eliminated and the organic upper phase containing fatty acid methyl esters (FAME) was cleaned two more times with 1.5 mL of water that was eliminated thereafter. FAME were recovered and analyzed by gas chromatography using a GC (Varian CP3800) with auto-sampler equipped with both polar and apolar capillary columns (ZB-WAX, 30 m length × 0.25 mm i.d. × 0.25 µm film thickness and ZB-5 30 m length × 0.25 mm i.d. × 0.25 µm film thickness), and with a splitless injector and a FID detector. The carrier gas was H_2, at a constant flow of 2 mL min^{-1}. FAME were identified and quantified by means of a standard 37-component FAME mix and other standard mixtures from marine bivalves combining analysis on polar and non-polar columns (Soudant *et al*. 1995). Non-methylene-interrupted (NMI) fatty acids 20:2n-5,11, 22:2n-7,13, 20:2n-5,13 and 22:2n-7,15 (also designated 20:2i, 22:2i, 20:2j and 22:2j in the literature) were identified in mussel lipids based upon retention times.

FA were expressed as the molar percentages of total FA content in algae total lipids and in mussel digestive gland NL and PL fractions

Furthemore, some FA indices were considered, the unsaturation index (PUI) and peroxidation index (PI), calculated as described by Hulbert *et al*. (2007):

Unsaturation index (PUI) = 1 × (% monoenoics) + 2 × (% dienoics) + 3 × (% trienoics) + 4 × (% tetraenoics) + 5 × (% pentaenoics) + 6 × (% hexaenoics).

Peroxidation index = 0.025 × (% monoenoics) + 1 × (% dienoics) + 2 × (% trienoics) + 4 × (% tetraenoics) + 6 × (% pentaenoics) + 8 × (% hexaenoics).

Figure 18. Fatty acid separation (Neutral and Polar lipid) through a silica microcolumn (left) and evaporation of lipid extraction to dryness under nitrogen conditions (right).

3.4. Mussel reproductive state

3.4.1 Sexual Maturity Index

Gonadal development was established for 15 mussels. Dissected mantle tissues were preserved in a 10 % v/v formaldehyde solution for fixation during at least 24 h, and were subsequently processed for histology. Dehydration of tissues was carried out in successive and increasing alcohol baths, followed by the replacement of alcohol for Ultraclear as clearing agent. Then, tissues were embedded in paraffin, cut at 3 µm, and stained with haematoxylin and eosin. Histological sections were observed under an optical microscope to identify sex and stage of gonadal development. Five stages were considered (Kim *et al.*, 2006): Stage 0, inactive gonad; Stage 1, gametogenesis has begun although no ripe gametes are visible yet; Stage 2, ripe gametes are present and gonias occupied about one-third of the section; Stage 3, gonad increased in area to about half the fully ripe condition; Stage 4, gonad fully ripe. A reproductive index, the sexual maturity index (SMI) was calculated from the average gonadal development stage of a mussel group.

3.4.2 Reproductive potential

A semi-quantitative of mussel reproductive potential was estimated by the *Total Reproductive Potential* (TRP) which was addressed from the calculation (SMI × GSI), being GSI the gonadosomatic index and SMI the sexual maturity index (used in Chapter 1). In Chapter 4 a quantitative index was also considered, the *Reproductive Potential*

(RP), which was performed after processing images obtained from histological slide. In this case, the proportion of gonadal tissue that is comprised of follicles containing developing or ripe gametes, which is reported as the "gamete volume fraction" (GVF), was quantified. For any individual mussel, the GVF can vary between 0%, for a reproductively inactive mussel, and 100%, for a mussel showing maximal reproductive development (Newell et a1., 1982). Mussel GVF was measured quantitatively using the IMAGE TOOL image analysis software and an Olympus BX-41 microscope, on eight randomly chosen areas. Since the gonad of *Mytilus* is homogenous with respect to the distribution of germinal cells and gametes, the tissue sections examined were representative of the whole gonad. The color image acquired was analyzed after adjusting the threshold by sampling area screen objects set by the operator. A threshold is a set of intensity values that separates pixels of interest from the rest of the image. The percentage of the mantle volume occupied by gametes was calculated from the relationship between the number of black pixels (represented by the gametes) and the total pixels in white.

The RP was calculated from the percentage of GVF and the mantle dry weight considering an standardized individual of 1 gram of total dry weight (MDW), RP=GVF × MDW_{st}, where MDWst was considered the ratio between the mantle and total meat dry weights.

4. Biomarkers to assess pollution effects

4.1. Immunological biomarkers

For hemolymph extraction, mussels were opened and the hemolymph of each individual was withdrawn from the adductor muscle using a 1 mL plastic syringe (Figure 19). Collected hemolymph was held on ice to reduce cell clumping. Each individual´s hemolymph sample was microscopically examined to eliminate samples which contain gametes or tissue debris and then were filtered in 80 μm mesh and conserved in a 1.5 mL vial that was hold on ice until analysis.

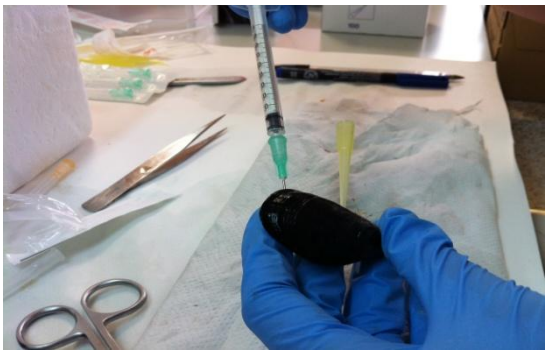


Figure 19. Hemolymph extraction.

Analyses were performed in 5 mL flow cytometer tubes (Falcon; BD BioSciences) to undergo the following analyses with a BD FacsVerse flow cytometer. All analyses were run for 30 s.

4.1.1. Total hemocytes, differential counts and viability

A 50 µl aliquot of hemolymph was transferred into a tube containing a mixture of Anti-Aggregant Solution for Hemocytes, AASH (Auffret and Oubella., 1995) and filtered sterile seawater (FSSW), 100 and 50 µl, respectively.

Two main sub-populations were distinguished according to size and cell complexity (granularity). Granulocytes are characterized by high FSC and high SSC, while hyalinocytes by high flow cytometer forward scatter (FSC) and high side-scatter (SSC) (Haberkorn *et al* 2014). Total hemocyte, granulocyte and hyalinocyte concentrations were estimated from the flow-rate measurement of the flow cytometer (Marie *et al*., 1999) as all samples were run for 30 s.
Total hemocytes, granulocyte and hyalinocyte concentrations were expressed by a number of cells by milliliter. Hemocytes were stained with SYBR Green I (Molecular probes, Eugene, Oregon, USA, 1/1000 of the DMSO commercial solution), and propidium iodide (PI, Sigma, St Quentin Fallavier, France, final concentration of 10 µg ml^{-1}) in the dark at 18 ºC for 120 min before flow cytometric analysis. PI permeates only hemocytes that lose membrane integrity and are considered to be dead cells, whereas SYBR Green I permeates both dead and live cells. The percentage of viable hemocytes was calculated by the ratio between the number of hemocytes not showing fluorescence (PI) and the number of total cells (SYBR green) × 100.

4.1.2. Phagocytosis

The phagocytosis assay used was adapted from Haberkorn *et al*. (2014). A 100 µl aliquot of hemolymph, diluted with 100 µl of FSSW, was mixed with 30 µl of Yellow-Green, 2.0-µm fluoresbrite microspheres (Polysciences, Eppelheim, Germany), diluted to 2 % in FSSW and was incubated for 120 min at 18 °C. Hemocytes were analyzed by flow cytometry on the FL1 detector (515–545 nm) to detect hemocytes containing fluorescent beads. Phagocytosis rate was calculated as the percentage of hemocytes that have ingested three fluorescent beads or more (=active hemocytes), while phagocytosis capacity was estimated as the average number of beads engulfed by active hemocytes (Hégaret *et al*., 2003).

4.1.3. Hemocyte Reactive Oxygen Species Production

Reactive oxygen species (ROS) production by hemocytes was measured using 2,7-dichlorofluorescein diacetate, DCFH-DA (Lambert *et al*., 2003). A 50 µl aliquot of hemolymph was diluted with 300 µl of FSSW. Four µl of the DCFH-DA solution (final concentration of 0.01 mM) was added to each tube and then incubated at 18°C for 120 min. After incubation, DCF fluorescence of hemocytes was measured by flow cytometry on the FL1 detector (515–545 nm). Results are expressed as the mean

geometric fluorescence (in arbitrary units, AU), quantitatively related to the ROS production of hemocytes.

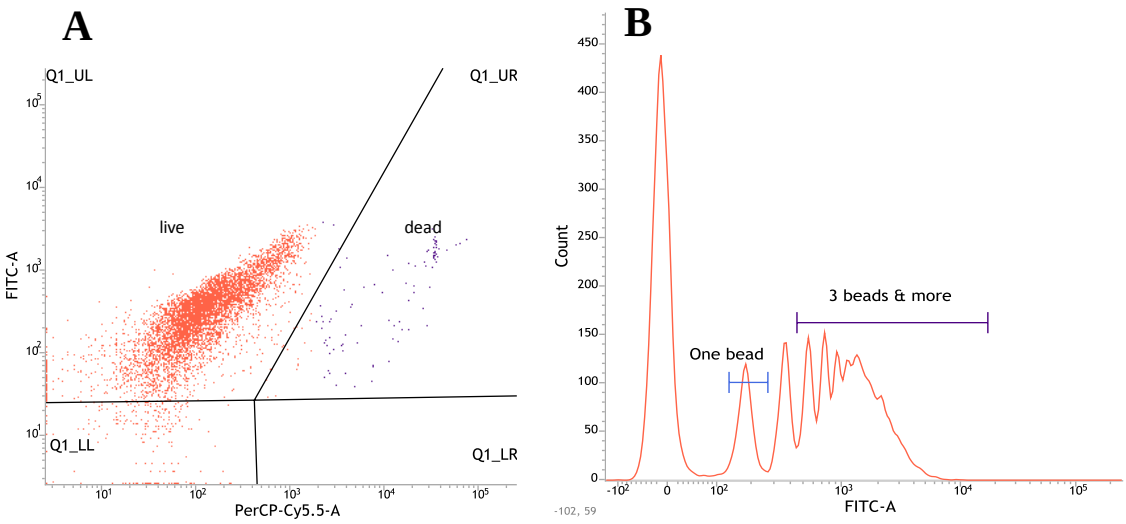


Figure 20. Graphic representation of mussel´s hemocyte viability (A) and hemocytes phagocytosis fluorescence (B) obtained by flow cytometry analysis.

4. 2. Physiological biomarkers

4.2.1. Physiological set-up

Physiological measurements were taken after the 24 h acclimatization period according to the procedures described by the ICES for the use of mussel scope for growth (SFG) as pollution biomarker (Widdows and Staff, 2006), with the modifications proposed by Albentosa *et al*. (2012a). Mussel experimental chambers were connected to an open-flow system for 24 h under controlled temperature and feeding conditions (15 ºC and 0.55 mg L^{-1} organic matter obtain from *I. galbana*, clone T-ISO, and 0.24 mg L^{-1} inorganic matter obtain from previously ashed marine sediment). Particulate concentration was measured with a Coulter Multisizer III fitted with a 100 µm orifice diameter tube.

4.2.2.. Clearance rates

Mussel clearance rate (CR, expressed in L ind^{-1} h^{-1}) was defined as the volume of water cleared of suspended particles per hour and was estimated from the difference between inlet and outlet concentrations in flow-through chambers according to the equation $CR=f\ (C_i–C_o)\ /\ C_i$ (Hildreth and Crisp, 1976), where f was the flow of water expressed in L h^{-1}, C_i was the inlet concentration and C_o was the outlet concentration, both expressed in particulate volume units, mm^3 L^{-1}. Ingestion rate (IR, mg h^{-1}) was estimated as the product of CR and the particulate organic matter (POM, mg L^{-1}). POM was determined as the difference between total particulate matter (TPM) and particulate

inorganic matter (PIM). TPM (mg L^{-1}) was determined as the dry weight (24 h at 100 ºC) of the suspension filtered and PIM was given as the weight remaining after ignition (1 h at 450 ºC).

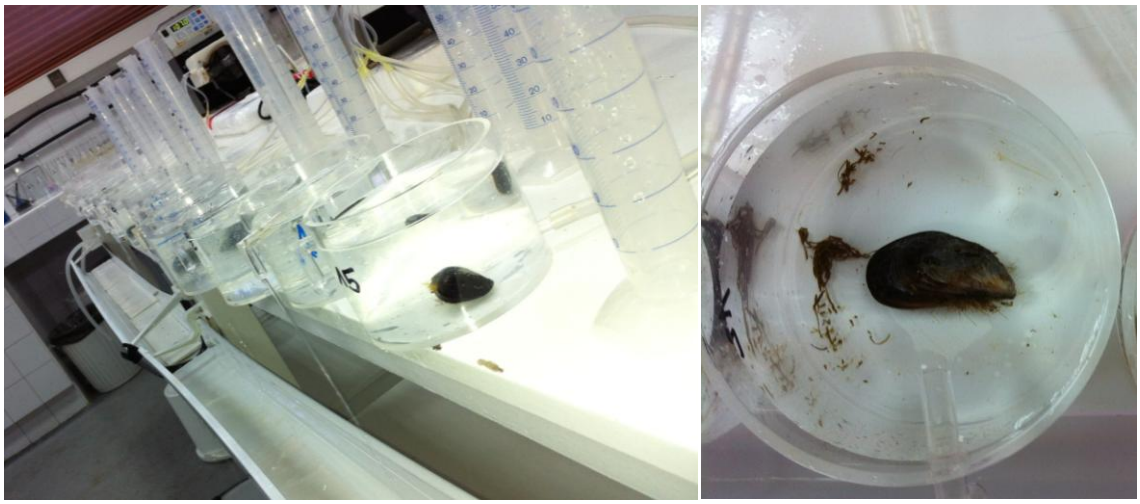


Figure 21. Open-flow physiology system (left) with individual containers (right). This system allows determining the clearance rate of each individual.

4.2.3.. Absorption rates

Absorption efficiency (AE, %) was calculated from the percentage of organic matter in the food and in the faeces according to Conover's ratio (1966), AE=((F−E)/([1–(E) F])x100, where F is the percentage of organic matter (ash-free dry weight) in the food and E is the percentage of organic matter in the faeces. Food organic content was estimated by filtering inlet samples through previously rinsed, ashed and weighed Whatman GF/C filters. The filters were then rinsed with ammonium formiate solution (0.5 M), dried for 24 h at 100 °C and ashed at 450 °C for 1 h. Faeces produced by each individual were filtered into Whatman GF/C filters and processed in a similar way to that described for food samples. Absorption rate (AR, mg h^{-1}) was obtained by multiplying the ingestion rate by the absorption efficiency (AR=IR x AE).

4.2.4. Respiration rates

The respiration rate (RR, mg O_2 ind^{-1} h^{-1}) was defined as the quantity of oxygen consumed by mussels and was calculated from the difference in oxygen concentration over a given period of time according to the equation $RR=[[(O_{2\,bl}-O_{2exp})/t]] \times V$, where O_{2bl} and O_{2exp} were the oxygen concentrations in the blank and experimental respirometers (Figure 22), respectively, expressed in mg O_2 L^{-1}; t was the time, expressed in h; and V was the volume, expressed in L.

Figure 22. Respiration system with individual respirometer´s containers.

4.2.5. Standardization of physiological rates

CRs were standardized to weight (b=0.67, Bayne and Newell, 1983) and to length (b=1.7, Filgueira *et al*., 2008). In any case, standardized clearance rates (CR_{st}) were calculated as follows: $CR_{st}=(S_{st}/S_{exp})^{b} \times CR_{exp}$, where S_{st} and S_{exp} are the standardized (to weight or to length) and experimental sizes, respectively, CR_{exp} is the measured CR and *b* is the corresponding allometric exponent. RRs were weight-standardized for a 1 g dw specimen using the allometric exponent b=0.75 (Vahl, 1973).

4.2.6. Energy Balance

Physiological rates were transformed to their energetic units previously to their integration in the energy balance equation. The following energy equivalents were used: 23 J mg^{-1} POM obtained from Widdows and Johnson (1988); 1 mg oxygen was equivalent to 0.6998 mL oxygen (Ansell, 1973); and 1 mL oxygen was equivalent to 20.33 J (Widdows and Johnson, 1988). SFG was calculated from the energy balance equation according to the expression SFG=(IR × AE)−R, where IR was the consumption of the energy available in the diet, AE was the absorption efficiency and R was the energy consumed by respiration. Energy lost via excretion was not included in the above equation because it accounted for less than 5% of the acquired energy (Bayne and Newell, 1983).

4.3. Biochemical biomarkers

Digestive glands were homogenized (1:4, w/v) in K-phosphate buffer 100 mM, pH 7.0 containing 0.15 M KCl, 1 mM DTT and 1 mM EDTA. Samples were centrifuged at 10.000 × g for 20 min to obtain a cytosolic fraction. In some cases, samples were centriguged again at 36.000 × g for 60 min to obtain a microsomal pellet (microsomal fraction) which was separated from the supernatant (cytosolic fraction) and resuspended in approximately 0.5 mL of microsomal buffer (50 mM Tris–HCl pH 7.6, containing 20% glycerol, 1 mM DTT and 1 mM EDTA). Cytosolic fractions were used for enzyme determinations and LPO analysis (Chapter 3) whereas microsomal fractions were used for LPO analysis (Chapter 1, 2 and 4).

4.3.1. Superoxide-dismutase (SOD)

SOD was measured through the "SOD-Assay kit-WST" (sigma 19160 SOD determination kit) . SOD was measured by the inhibition of SOD caused by the reduction of O_2 in presence of Xanthine-oxidase (XO) at 450 nm and expressed as U min^{-1} mg $protein^{-1}$, being U defined as units of SOD that is the amount of enzyme which inhibits 50% of reaction.

4.3.2. Catalase (CAT)

CAT was measured according to Claiborne. (1985) by the decrease in absorbance at 240nm by H_2O_2 consumption (extinction coefficient, ε = -0.04 mM^{-1} cm^{-1}). For this activity, H_2O_2 (30 % volume) was used as substrate of the reaction at 50 mM in a k-phosphate buffer 50 mM, pH=7.0 solution. CAT was expressed as µmol of H_2O_2 degraded min^{-1} mg^{-1} protein.

4.3.3. Glutathione reductase (GR)

GR activity was measured according to Ramos-Martinez *et al*. (1983) by following the decrease of the absorbance at 340 nm (ε = -6.22 mM^{-1} cm^{-1}) due to the oxidation of NADPH produced during the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH). Reaction medium consist of k-phosphate buffer 100 mM, pH=6.8, GSSG 1 mM and NADPH 0.12 mM at final concentration. GR was expressed as nmol min^{-1} mg^{-1} protein.

4.3.4. Glutathione peroxidase (GPx)

GPx activity was measured according to Livingstone *et al*. (1992) by following the decrease of the absorbance at 340 nm (ε = -6.22 mM^{-1} cm^{-1}) due to the oxidation of NADPH produced during the formation GSH from GSSG. This reaction has a medium of K-phosphate buffer 50 mM, pH=7.5, GSH 3.5 mM, commercial glutathione reductase 1U, NADPH 0.12 Mm and sodium azide 1 mM at final concentration. GPx was expressed as NADPH oxidized min^{-1} mg^{-1} protein.

4.3.5. Glutathione S-transferase (GST)

GST was measured according to Habig *et al*. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, compound recognized by the isoenzymes of the GST. The assay was carried out at 340 nm (ε = 9.60 mM^{-1} cm^{-1}) in 100 mM k-phosphate buffer pH=6.5, 60 mM, CDNB and 10 mM GSH at a final volume of 345 µL. The measurement consist of quantifying the formation of S-2,4-dinitro fenil glutathione conjugated from CDNB for 4-5 min. GST activity was expressed as nmol min^{-1} mg^{-1}.

4.3.6. Lipid peroxidation (LPO)

LPO was quantified on gland cytosolic and microsomal fractions measuring the absorbance generated by thiobarbituric acid reactive substances (TBARS), following Buege and Aust (1978). TBA reagent was a dissolution of hydrochloric acid (HCL)

0.25 N with 15 % of trichloroacetic acid and 0.375 % of tiobarbituric acid. 100 µL of diluted sample was added to 100 µL TBA reagent and kept in a water bath at 80 ºC for 20 min. Samples were then centrifuged to separate the liquid phase from the pellet. The malondialdehde (MDA) formed was estimated at 535 nm (ε = 156.10 M^{-1} cm^{-1}) using a standard of malonaldehyde bis-(dimethylacetal). LPO was expressed as nmol MDA mg^{-1} prot.

Figure 23. Water bath for LPO samples.

Protein concentrations in cytosolic and microsomal fractions were measured according to Lowry *et al*. (1951) by using bovine serum albumin as standard. 100 µL of sample was mixed with a medium which include sodium-potasium tartrate, copper (II) sulphate pentahydrated ($CuSO_4$ $5H_2$), sodium carbonate ($NaSO_3$) and sodium hydroxide (NaOH) at a final volume of 5 mL. The sample was incubated 30 min in darkness and 0.5 mL of Folin reagent (diluted × 2) were added and samples were incubated again for 30 min in darkness before measuring the absorbance at 595 nm.

4.4. Biaccumulation

4.4.1.. Metals

For simplicity, the term metal is used without distinction between the target metals (Cd, Cu, Hg, Pb and Zn) and metalloid (As). Samples for metals analyses were digested with nitric acid (Suprapur, Merck) in a microwave oven (Besada *et al*., 2011). Briefly, freeze-dried mussel samples were placed in a high pressure Teflon reactor, and after the addition of the nitric acid, digested in a microwave oven at 90 ºC for 10 min and then at 180 ºC for 60 min. A Perkin-Elmer AAnalyst 800 spectrophotometer, equipped with a Zeeman background correction device (Cd, Pb and As by electrothermal AAS) was used throughout. Total Hg was determined by the cold vapour technique, employing a Perkin-Elmer FIMS-400 system ($SnCl_2$ as reducing agent).

Detection limits were 0.003, 0.005, 0.050, 0.50, 0.30, 0.30 mg kg^{-1} dry weight (mg kg^{-1} dw) for Hg, Cd, Pb, Cu, Zn and As, respectively.

4.4.2. Polycyclic aromatic hydrocarbons

4.4.2.1. Total PAHs

PAH in mussel samples (wet tissues) were Soxhlet extracted with a 3:1 hexane:acetone mixture and analysed by high performance liquid chromatography (HPLC) as described elsewhere (Soriano *et al*., 2006). In summary, samples to be analysed by HPLC were submitted to a clean-up step by column chromatography on 10% deactivated alumina and hexane elution. Twelve PAHs (phenanthrene, anthracene, fluoranthene, pyrene, chrysene, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene, dibenz[ah]anthracene and benzo[ghi]perylene) were determined by HPLC (HP 1100 apparatus, Agilent Technologies) coupled with a wavelength programmable fluorescence detector (HP 1036, Agilent Technologies), using a ZORBAX Eclipse PAH column (Agilent Technologies), eluted with a methanol:water gradient. Detection limits were in the range of 0.1 (phenanthrene) to 0.4 µg kg^{-1} dw (indeno[1,2,3-cd]pyrene).

4.4.2.2. Fluoranthene (FLU)

Fluoranthene quantification in mussel´s tissues were conducted in two different ways in order to quantify fluoranthene accumulation in total mussels tissues using 3 pools of 5 individuals and individually in the same individuals used for biomarker analysis (digestive glands, n=9) in order to check the fluoranthene effect on biomarker´s responses according to the diet supplied (Chapter 3).

4.4.2.2.1. FLU in total mussel tissues

A solution of FLU in acetonitrile was obtained from Dr. Ehrenstorfer (Augsburg, Germany). Anhydrous sodium sulfate for analysis was obtained from Merck and MP Alumina B Super I from MP Biomedicals for column chromatography; both were activated at 400 °C overnight. The analytical method for the quantification of FLU in mussel tissues has been described elsewhere (Soriano *et al*. 2006; Viñas Diéguez *et al*. 2002). In brief, a portion of 2–4 g of mussel homogenate tissue was Soxhlet extracted with a 1:3 acetone:hexane mixture for 10 h. Samples to be analyzed by high performance liquid chromatography (HPLC) were submitted to a clean-up step by column chromatography on deactivated alumina (10% water) and hexane elution. FLU concentrations were determined by HPLC (HP 1100 apparatus, Agilent Technologies, Palo Alto, CA, USA) coupled with a wavelength programmable fluorescence detector (HP 1036, Agilent Technologies, Palo Alto, CA, USA). The column (Zorbax Eclipse PAH, Agilent Technologies, CA, USA) was kept at 23.5±0.1 °C and eluted with a methanol:water gradient, starting with 30% methanol (0–0.5 min), then increasing the methanol content to 80% (0.5–5 min), and finally to 100% methanol (5–62 min) that was held for 1 min. After this gradient, two steps, one for cleaning with a mixture of methanol–acetone (1:1) for 11 min and another one for reconditioning the column with 30% methanol, were carried out.

A certified solution, supplied by Dr. Ehrenstorfer was employed in the quantification, using a multilevel calibration at six points between 25 and 350 µg kg^{-1} for FLU. 2-Methylchrysene was employed as an internal standard. The analytical method was subject to a continuous external quality control process by the participation in the Quality Assurance of Information for Marine Environmental Monitoring in Europe exercises and to an internal quality control by means of analyzing an internal reference material (IRM) with each set of samples.

Bioaccumulation of exposed mussels was evaluated by means of FLU concentrations in total mussel tissues expressed as µg g^{-1} dry weight of mussels, and by the accumulation efficiency expressed as % of FLU accumulated with respect to the total FLU offered to the mussels.

4.4.2.2.2 FLU in mussel´s digestive gland

Fluoranthene was quantified in the digestive gland of individual mussels (n=9) using a Stir Bar Sorptive Extraction-Thermal Desorption-Gas Chromatography-Mass Spectrometry (SBSE-TD-GC-MS) method adapted from Lacroix *et al*. (2014). Briefly, 50 mg wet weight (w.w.) of tissue powder were digested by saponification and FL was extracted using polydimethylsiloxane stir-bars (Twister 20 mm × 0.5 mm, Gerstel). Bars were subsequently analysed using a 7890 Agilent GC system coupled to an Agilent 5975 mass spectrometer (Agilent Technologies) and equipped with a Thermal Desorption Unit (TDU) combined with a Cooled Injection System from Gerstel. Thermodesorption and GC-MS conditions were as detailed in Balcon *et al*. (2011) except the oven program of temperature that was: from 50 °C (1 min) to 200 °C at 30 °C min^{-1}, and then to 320 °C (2 min) at 10 °C min^{-1}. FLU was quantified relatively to [$^{2}H_{10}$]-FLU using a calibration curve ranging from 1 ng to 10 µg per bar. Results are expressed as µg FLU g^{-1} ww. The limit of quantification (LOQ) was calculated by the calibration curve method (Shrivastava *et al*, 2011) and was 0.2 µg g^{-1} ww.

4.4.3. Polybrominated diphenyl ethers

For the analysis of PBDE congeners 28, 47, 66, 85, 99, 100, 153, 154, 183 homogenised tissue of mussels was extracted in a Soxhlet using a solvent mixture of n-hexane:acetone (3:1), spiked with appropriate recovery standard (BDE-77). The clean-up was performed using an alumina column followed by silica gel column. The concentration of the BDEs congeners were determined by GC-MS using an Agilent 6890N coupled to an Agilent 5973N mass selective detector operated in negative chemical ionization (NCI) mode. A CPSil8 CB (30 m × 250 µm i.d. × 0.25 µm film thickness) capillary column was used. The injector temperature was 275 ºC and the oven temperature program was 90 ºC held for 3 min, ramped to 210 ºC at 30 ºC min^{-1}, held for 20 min, ramped to 290 ºC at 5 ºC min^{-1}, held for 17 min and to 310 ºC at 35 ºC min^{-1} and held for 5 min. The mass selective detector with quadrupole analyser was operated in the selected ion-monitoring mode (SIM). The monitored ions (m/z) were 79, 81, 159 and 161 for all congeners. For the internal standard (octachloronaphtalene) the ion (m/z) was 403.80. Methane was used as reagent gas. The MS transfer line

temperature was held at 290 ºC, the MS source and quadrupole temperatures were 200 ºC and 106 ºC respectively. Detection limit was 0.001 µg kg^{-1} ww.

4.4.4. Polychlorinated biphenyls and organochlorinated pesticides

The determination of the seven PCBs recommended by ICES (Σ_7PCBs, congeners 28, 52, 101, 118, 138, 153, 180), hexachlorocyclohexanes HCHs (sum α-HCH and γ-HCH), dichlorodiphenyl trichloroethane DDTs (sum p,p'-DDE, p,p'-DDD, p,p'-DDT and o,p'-DDT) and chlordanes (sum cis- and trans-chlordanes), was carried out following a previously described method (De Boer, 1988; González-Quijano and Fumega, 1996), based on a Soxhlet extraction using a solvent mixture of n-pentane:dichloromethane (1:1) for 8 h. An aliquot of the Soxhlet extract was used to determinate gravimetrically the lipid content. The chlorinated hydrocarbons were removed from the lipids by alumina column chromatography followed by separation of PCBs from the chlorinated pesticides using silica column chromatography. Concentration levels were determined by gas chromatography with electron capture detector (GC-ECD) with capillary columns (50 m, 0.25 mm i.d. and 0.25 µm film) and He as carrier gas. Quantification was performed using multilevel calibration curves obtained by injection of standard solutions of seven different levels of concentration. Detection limit was 0.01 µg kg^{-1} ww.

4.4.5. Chemical Pollution Index

CPI was calculated for each site, to summarize the chemical data, as the average of the ratios between the pollutant concentrations (C) in each site and the corresponding environmental quality criterion (Ccrit) for each analyte (Bellas *et al*., 2011): $CPI=\sum_i[\log(C_i/ Ccrit_i)]$. A principal component analysis (PCA) was carried out with the autoscaled data to obtain the most relevant variables in the CPI data set. According with Beiras *et al*. (2012), only those chemical showing loading >0.7 were consider in the CPI calculation. Quality criterion used were: OSPAR BAC (OSPAR Commission, 2010b) for Cd, Hg, Pb, PCBs and HCHs, the environmental quality standard (EQS) in biota proposed by the European Commission was used for BDEs (OSPAR Commission, 2012b), and the Norwegian level I (Green *et al*., 2012) for DDT, benzo[a]pyrene (B[a]P) and the rest of metals analyzed (Cu, Zn and As). The rest of pollutants have not been considered due to the inexistence of environmental quality criterion. (B[a]P) was considered as the most representative PAH due to its high toxicity and persistence.

5. Statistical analysis

In general, data of this thesis was statistically analyzed using mainly STATGRAPHICS CENTURION XV, although statistical analyses of fatty acid data were performed with the PRIMER v6 software.

Data normality and homogeneity of variances (Levene's test) was checked before statistical analyses were performed. When requirement of normality and homogeneity of variances were not met, transformation of data was performed through arcsine square root and log transformations.

5.1. Student-t test

This test was used to compare variables between two groups. In this thesis, groups considered were: *treatments* (Exposed and non-exposed) and *dietary conditionings*. This statistical analysis was used in Chapters 1, 3 and 4.

5.2. One-way and two-way Analyses of Variance (ANOVA)

Two-way-ANOVA was performed to establish significant differences between three or more condition groups (for example: mussel nutritive state (NS) and pollution exposure treatments). One-way-ANOVA was performed to assess differences between exposure treatments and between condition groups in Chapters 2 and 4. A LSD pos hoc test was used to test differences among groups with $p<0.05$ as the significance level for all the analysis. Non-parametric analyses (Kruskal-Wallis test) were also performed when the variables did not fit the requirements of ANOVA.

5.3. Analyses of Covariance (ANCOVA)

ANCOVA was performed to assess the behaviour of biomarker responses in presence of FLU according to the two dietary conditioning from Chapter 3. ANCOVA evaluates whether values of a dependent variable (biomarker considered) are equal across levels of an independent variable (FLU concentration), while controlled by a categorical factor (dietary conditioning). To carry out this statistical analysis, the FLU accumulation and biomarker values of each individual (mussel) were considered. This analysis provided information about i) the effect of toxicant (regression line fit) in a biomarker response, ii) differences of biomarker values between dietary conditions (comparison of intercepts between the two regression lines) without the presence of FLU (Non-exposed mussels) and iii) the variability of biomarker responses to increasing FLU concentration according to dietary conditioning (comparison of slopes between the two regression lines); with $p<0.05$ as the significance level for all the analysis.

5.4. Principal Component Analyses (PCA)

PCA was used to obtain a reduced number of new variables (principal components) that result from the linear combinations of the original variables, and allow selecting the most relevant variables in the data set in Chapters 1 and 3. The autoscaling of the data was performed to obtain the same range of normalized data in the PCA. For that, each value was calculated from: $x= (x_i-\bar{x})/\sigma$, where x is the new data, x_i is the initial data, $\bar{x}$ is the average of the data and σ is the standard deviation of the data. PCA analyses were carried out in order to establish relationships between biomarkers responses and biological/chemical parameters. According to Peterson (2000) a factor loading ≥0.32, corresponding to an explained average variance of 56.6%, is recommended. A threshold factor loading of 0.40 was selected for a good association between variables and the component whose cumulative variance explained around 60% was considered.

5.5. Mantel´s test

Mantel´s test analysis (Mantel and Valand, 1970) was performed in order to obtain an overall view of the relationship between biomarkers responses with biological and pollution parameters in the field in Chapter 1. Mantel´s test is a statistical test of the correlation between two square matrices (distances matrices), which summarizes pairwise similarities among sites. For this analysis, 4 distance matrices were performed (biological variables, chemical bioaccumulation, physiological biomarkers and biochemical biomarkers).

5.6. Integrated Biomarker Response (IBR)

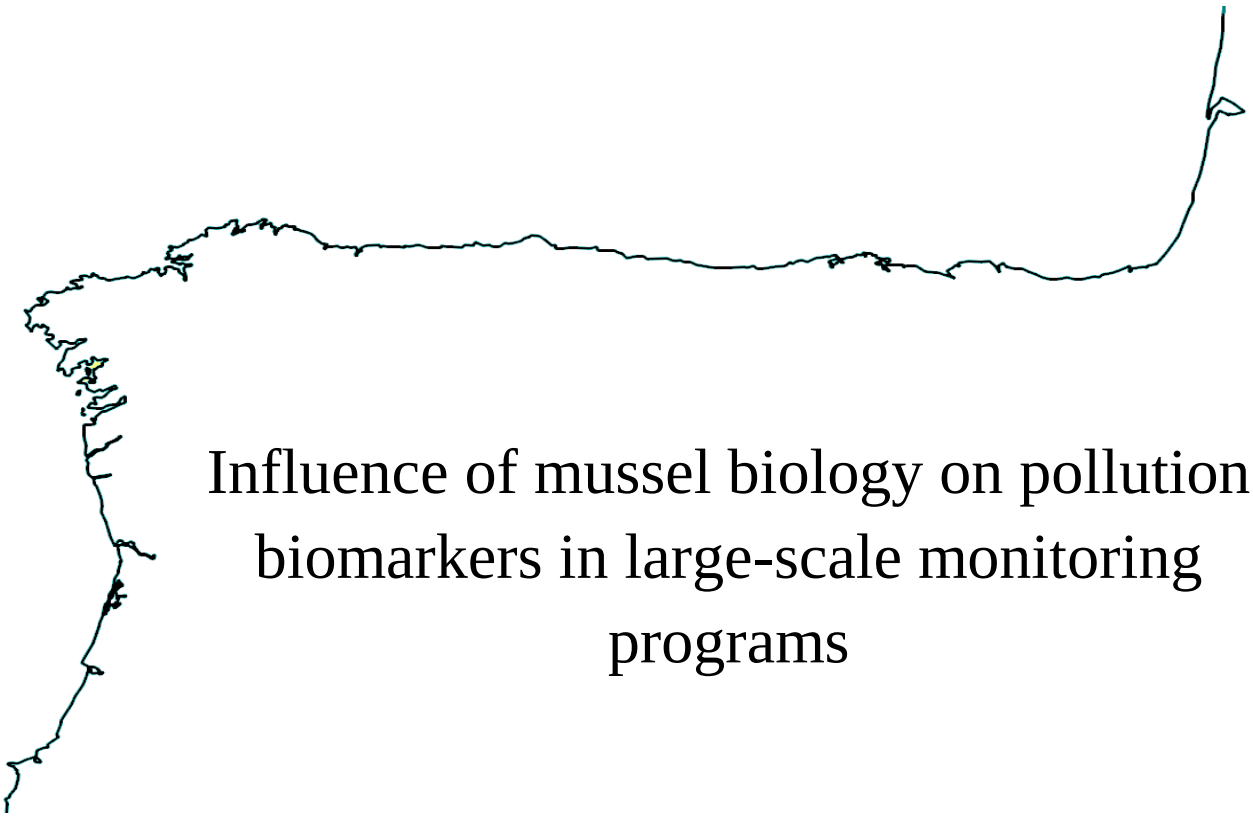
Mussel integrative biomarker responses were evaluated through the IBR (Beliaeff and Burgeot, 2002, revisited by Devin *et al*., 2013). This index was used in order to integrate the mussel biomarker levels considering the reproductive cycle (Chapter 4). Only biomarkers showing a significant effect of either pollutant or reproductive cycle were included.

5.7. Analyses of Fatty Acid data

FA data from Chapter 3 were statistically analyzed by different ways. Firstly, a similarity percentages analysis (SIMPER) was performed on the relative FA percentage data to describe the differences in FA classes among diets. SIMPER identifies the FAs that contribute most to the variations in the assemblage patterns recorded. Only the FAs that cumulatively contributed up to 80% of the dissimilarities recorded were selected to characterize the differences in the FA profile of mussels fed with different diets (Clarke and Gorley, 2006). For that, the data on the relative FA percentage of the samples was logarithmically (log [x + 1]) transformed and converted into a Bray-Curtis similarity matrix to start the multivariate analyses. Afterwards, the Bray-Curtis similarity matrix was used for a 2-way ANOSIM to test whether samples within NL or PL classes clustered by diet for each in mussel´s digestive gland tissue. ANOSIM calculates a

global R statistic that assesses the differences between groups, R = 1, R = 0.5 and R = 0 indicate a ‘perfect’, ‘satisfactory’ and ‘poor’ separation of the clusters, respectively (Clarke and Gorley, 2006). This analysis only gave a general picture of the samples, grouping them according to the main explicative FA, and statistics only considered the specific FA obtained. Thus the most representative groups of FA (20:5n-3, 22:6n-3, NMI, SFA, MUFA and PUFA) were analyzed in mussel´s digestive gland NL and PL separately though a single ANOVA analysis, in order to check the effect of FLU in these FA and groups of FA, in detail. The relative FA percentage was statistically analyzed using software PRIMER V6.

CHAPTER 1



Influence of mussel biology on pollution biomarkers in large-scale monitoring programs

Carmen González Fernández, 2016

The research presented in this chapter forms part of the following publication:

González-Fernández, C., Albentosa, M., Campillo, J. A, Viñas, L., Fumega, J., Franco, A., Besada, V., González-Quijano, A., Bellas, J., 2015. Influence of mussel biological variability on pollution biomarkers. Environ. Res. 137C, 14–31.

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IF: 4.033

ABSTRACT

This study deals with the identification and characterization of biological variables that may affect some of the biological responses used as pollution biomarkers. With this aim, during the 2012 mussel survey of the Spanish Marine Pollution monitoring program (SMP), at the North-Atlantic coast, several quantitative and qualitative biological variables were measured (corporal and shell indices, gonadal development and reserves composition). Studied biomarkers were antioxidant enzymatic activities (CAT, GST, GR), lipid peroxidation (LPO) and the physiological rates integrated in the SFG biomarker (CR, AE, RR). Site pollution was considered as the chemical concentration in all tissues of mussels.

A great geographical variability was observed for the biological variables, which was mainly linked to differences in food availability along the studied region. An inverse relationship between antioxidant enzymes and the nutritional state of the organism was evidenced, whereas LPO was positively related to nutritional state and, therefore, with higher metabolic costs, with their associated ROS generation. Mussel condition was also inversely related to CR, and therefore to SFG, suggesting that mussels keep an "ecological memory" from the habitat where they have been collected. No overall relationship was observed between pollution and biomarkers, but a significant overall effect of biological variables on both biochemical and physiological biomarkers was evidenced. It was concluded that when a wide range of certain environmental factors, as food availability, coexist in the same monitoring program, it determines a great variability in mussel populations which mask the effect of contaminants on biomarkers.

Keywords: biomonitoring, mussels, physiological biomarkers, antioxidant biomarkers, biological variability, confounding factors

1. Hypothesis

Variability of natural environmental conditions affects biological processes of organisms which are used as biomarkers in marine monitoring programs. Biomarkers responses to the presence of pollutants might be masked by the variation of biomarkers levels due to different environmental conditions.

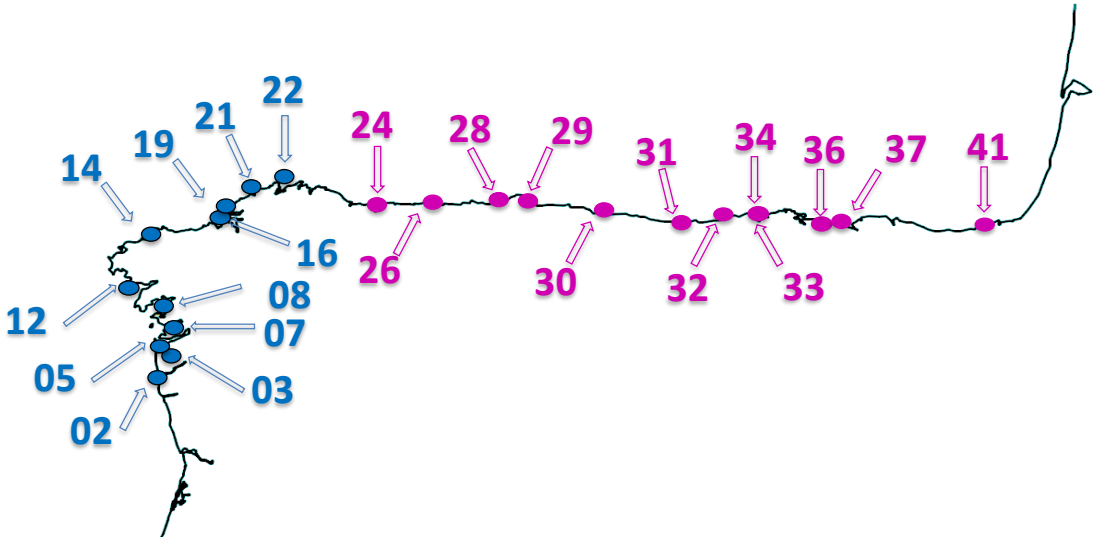
2. Objectives

The first objective of this Chapter was to identify and characterize quantitative and qualitative biological parameters in wild mussel populations (corporal and shell indices, gonadal development and reserves composition) which vary depending on environmental conditions in a large monitoring program as the Spanish Marine Pollution Program (SMP).

The second objective was to establish the relationships between those biological variables and biomarker responses and also between pollutants bioaccumulation and biomarkers, in order to describe to what extent the variation in biomarker responses can be explained by biological variables and pollution.

3. Summary of the design of the study

Monitoring of 23 sites along the N-NW Spanish coast, which are included in the Spanish Marine Pollution Program (SMP), were sampled in September of 2012. Sampling sites belong to the Atlantic coast (blue points) and Cantabrian coast (purple points)



4. Results

4.1 Biological variables

Table 3 shows mussel biometric parameters, total biochemical composition and sexual maturity indices of the 23 sampling sites. The biological characterization of mussels' populations shows that, in general, biological indices present a great variability between sampling sites.

Shell morphology showed certain variability between sampling sites. Shell indices L/H, L/W, H/W ranged from 1.6, 2.2 and 1.2 to 2.1, 2.8 and 1.7, respectively, presenting the H/W index the highest variability between sites. Shell morphology was clearly influenced by wave exposure, according to the semi-qualitative classification shown in Table 3. Mussels from highly dynamic environments had higher L/H indices ($r=0.742$, $p<0.001$) whereas mussels from sites less exposed to waves had higher H/W indices ($r=0.615$, $p<0.001$). Moreover, mussels with higher L/H indices had lower shell thickness, ST ($r=-0.5968$, $p<0.01$). ST ranged from 138.2 to 186.5 mg/cm^2 and it seems to be related to the HI ($r=0.489$, $p<0.05$).

Both condition indices (CI_{shell} and CI_{vol}), which are normally used as indicators of the physiological state of mussels, were positively correlated with gonad relative size and, to a lesser extent, negatively correlated with gill size. As mussel condition was mainly determined by gonad weight, variability observed in CI was basically a result of gonad relative size (GSI), which ranged from 0.03 to 19.46. GSI variability was higher than gill or gland variability and it was three times higher than condition variability. These relationships were more evident when using CI_{vol} ($r=0.846$ and $r= -0.668$, for GSI and GI, respectively) instead of CI_{shell} ($r= 0.597$ and $r= -0.3709$), as CI_{vol} reflects better the mussel nutritive state (Crosby and Gale, 1990). Therefore, further comparisons between mussel condition and biomarkers will be carried out with CI_{vol}.

The gonadal development of the mussels from each site is shown in Figure 24 where gametogenic stages, GSI and SMI values are shown. A high association between mantle size (GSI) and sexual maturation levels (SMI) ($r=0.892$, $p<0.001$) was observed, indicating that growth of mantle tissues runs parallel with gametogenesis. Anyhow, in none of the 23 sites there were mussels with high GSI and low SMI.

Other sources of biological variability were related to main biochemical components of mussel tissues (Table 3). In general, the condition index was positively related to the amount of carbohydrates in mussel tissues ($r=0.751$, $p<0.001$). Absolute reserves values ranged from almost 140 mg of carbohydrates per individual in Pontevedra-Raxó, to only 50 mg ind^{-1} in Suances. High-condition mussels were also characterized by a high development of their mantle tissues ($r=0.767$, $p<0.001$) and clearly presented an advanced stage of gametogenesis ($r=0.647$, $p<0.001$)

Table 3. Biological parameters (mean values and standard deviations) of mussels *(M. galloprovincialis)* from the N-NW Spanish coasts in November 2012. L: Length, H: Height, W: Width, ST: Shell Thickness, CI_{Shell}: Shell condition Index, CI_{Vol}: Volumetric condition index, GI: Gill Index, GSI: Gonadosomatic Index, HI: Hepatosomatic Index, TRP: total reproductive potential, SMI: sexual madurity index and CH: Carbohydrates for a standard individual of 8 ml of volume of internal cavity.

Nº	Sampling site	Biometric measurements												Biochemical measurements			
		L/H	L/W	H/W	ST	CI_{Shell}	CI_{Vol}	GI	GSI	HI	RI	TRP	SMI	Proteins	Carbohydrates	Lipids	CH
					mg/cm^2									mg/g organic matter			mg/ind
	Atlantic coast																
2	Oia	1.90±0.19	2.69±0.26	1.42±0.13	153±15	12.4±1.1	4.7±0.7	9.3±1.0	8.6±3.9	12.0±1.1	69.9±3.4	10.4±11.5	1.13±1.18	560±6	312±11	126±4	101.0
3	Samil	1.79±0.25	2.73±0.18	1.54±0.18	159±27	12.5±1.2	4.8±0.6	9.0±1.7	11.6±3.9	13.6±1.9	65.6±2.4	18.2±21.8	1.27±1.33	491±29	376±34	132±5	122.3
5	Cabo Home	2.11±0.12	2.48±0.18	1.18±0.14	155±18	11.6±2.0	4.9±0.7	10.8±2.1	13.7±8.0	13.9±2.2	61.4±6.6	43.7±38.4	2.40±1.59	508±14	371±15	120±1	128.4
7	Pontevedra Raxo	1.79±0.15	2.47±0.18	1.38±0.16	171±21	11.9±1.8	5.6±0.9	8.5±1.4	19.3±3.4	15.1±1.7	56.9±3.4	55.8±25.5	2.80±1.08	512±44	353±40	133±4	140.0
8	Chazo	1.76±0.11	2.64±0.19	1.50±0.13	170±27	12.3±2.6	5.5±1.0	9.6±2.0	19.4±6.3	15.1±2.2	55.7±4.6	57.6±35.3	2.93±1.14	599±7	301±12	98±19	117.0
12	Punta Insua	2.11±0.13	2.54±0.14	1.21±0.10	179±36	10.2±2.3	4.8±0.7	8.4±1.8	6.8±5.8	14.8±2.0	69.9±4.7	14.2±16.4	1.00±1.40	555±47	286±11	158±35	91.5
14	Corme	2.03±0.15	2.46±0.16	1.22±0.15	169±14	10.1±2.0	4.9±0.8	6.9±0.8	9.3±4.7	11.7±2.2	71.8±5.0	14.7±17.3	1.27±1.38	551±22	328±13	120±9	109.9
16	A Coruña TC	1.83±0.09	2.78±0.23	1.52±0.19	145±20	13.5±1.3	5.3±0.7	7.7±1.3	11.5±5.6	12.8±1.5	67.8±4.9	19.4±17.3	1.40±1.12	614±16	315±8	69±8	119.9
19	Ferrol Palma	1.82±0.12	2.74±0.13	1.51±0.12	143±11	14.1±1.3	5.5±0.6	8.7±1.4	11.5±5.7	12.2±1.5	67.4±5.3	30.5±23.9	2.36±1.52	676±36	221±24	101±11	85.3
21	Cedeira	1.66±0.11	2.81±0.14	1.70±0.13	154±18	12.8±1.3	4.9±0.6	3.0±1.2	7.9±6.0	12.9±1.9	70.2±4.6	11.1±20.8	0.80±1.26	610±42	271±24	117±17	94.7
22	Espasante	1.86±0.13	2.65±0.20	1.43±0.17	179±28	11.6±1.7	4.9±0.5	8.3±0.8	10.4±2.5	13.3±2.0	67.9±2.9	22.4±20.0	2.07±1.57	571±20	299±25	129±4	99.8
	Cantabrian coast																
24	Ribadeo	1.67±0.06	2.46±0.15	1.47±0.11	187±18	12.4±2.3	6.2±0.7	6.4±0.8	15.6±4.4	14.9±2.3	63.0±3.3	34.7±25.1	2.14±1.16	583±13	287±8	128±4	124.2
26	Luarca	1.86±0.44	2.38±0.38	1.36±0.40	169±26	10.4±1.4	4.5±0.6	8.5±1.5	9.5±6.4	13.8±1.9	68.0±5.5	23.9±26.7	1.67±1.58	650±38	216±21	133±17	65.5
28	Avilés	1.91±0.12	2.40±0.17	1.26±0.12	170±28	9.7±2.3	4.4±0.7	8.8±1.4	8.0±5.6	13.6±1.2	69.3±5.8	11.6±14.5	1.07±1.19	666±10	225±5	108±5	66.3
29	Gijón	1.92±0.13	2.58±0.17	1.35±0.16	174±19	10.0±2.1	4.7±0.8	9.0±1.4	7.1±3.3	12.3±1.7	71.4±4.5	6.7±8.0	0.80±0.94	643±13	201±6	155±7	65.0
30	Ridadesella	1.67±0.08	2.65±0.14	1.59±0.10	141±18	9.9±1.5	3.7±0.4	10.6±1.9	0.03±0.0	13.5±1.9	75.7±2.5	0.0±0.0	0.00±0.00	683±4	201±5	115±2	48.7
31	SV Barquera	1.63±0.09	2.57±0.19	1.57±0.16	175±22	10.7±1.5	4.8±0.5	9.4±1.0	10.9±3.1	13.1±1.3	66.4±2.8	15.8±20.5	1.20±1.52	672±1	197±2	130±3	64.2
32	Suances	1.72±0.08	2.61±0.17	1.52±0.13	165±18	8.7±1.3	3.9±0.6	10.8±1.9	3.1±3.8	14.2±1.8	71.7±4.7	4.6±7.6	0.50±0.91	672±24	191±22	136±2	50.9
33	Santander Pantalán	1.71±0.09	2.47±0.18	1.44±0.12	166±24	10.8±1.6	5.1±0.7	8.3±1.5	9.5±5.5	14.3±1.6	67.7±5.3	12.4±19.5	1.00±0.93	632±18	231±8	136±10	83.5
34	Santander Pedreña	1.78±0.07	2.72±0.16	1.53±0.13	155±25	10.5±1.4	4.4±0.4	9.8±1.2	6.8±4.0	13.6±1.6	69.6±3.9	5.5±8.6	0.53±0.83	634±3	230±4	135±1	69.0
36	Castro Urdiales	1.91±0.12	2.56±0.15	1.34±0.12	166±22	9.6±1.7	4.3±0.8	10.0±2.7	7.4±7.1	13.2±1.5	69.2±6.4	22.2±26.4	1.67±1.67	647±33	200±32	152±1	58.1
37	Bilbao Azcorri	1.93±0.16	2.69±0.21	1.39±0.15	166±27	9.5±2.1	4.3±0.7	10.2±1.3	3.2±5.0	13.4±1.2	73.0±4.9	2.5±7.2	0.20±0.56	564±20	309±5	126±25	89.7
41	Hondarribia	1.62±0.06	2.79±0.22	1.72±0.17	144±16	12.0±1.4	4.6±0.7	9.7±1.8	6.9±7.0	11.4±2.1	71.8±5.8	4.5±8.1	0.36±0.63	630±32	223±18	145±13	70.3

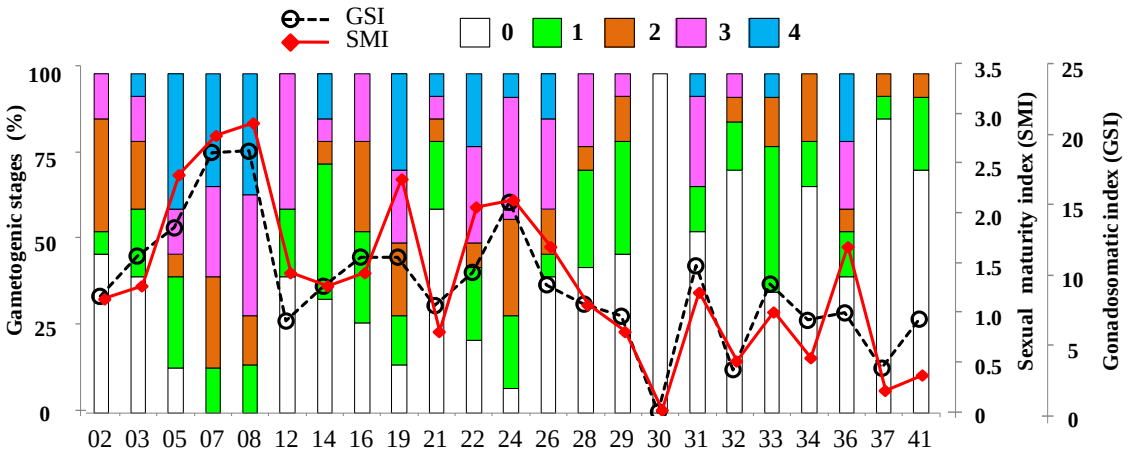


Figure 24. Gametogenesis stages, variation of gonadosomatic index (GSI) and sexual maturity index (SMI) of mussels (*Mytilus galloprovincialis*), from the 23 sampling sites along the two geographical areas (Atlantic and Cantabrian coasts) collected in November 2012.

According to the two geographical areas established: Atlantic (11 sites) and Cantabrian (12 sites) coasts, Student´s tests were performed with biological variables in order to study differences between areas (Table 4). In general, Atlantic mussels exhibited a significantly higher condition index ($t=2.48$, $p<0.05$) due to their higher GSIs ($p<0.005$). Atlantic mussels showed much higher sexual maturation levels, with almost 70% of mussels in stages 3 or 4. Therefore, SMI in Atlantic mussels was twice (1.8) the SMI in Cantabrian mussels (0.9), being this difference highly significant ($t=3.02$, $p<0.01$). As a consequence of this higher sexual development, the number of undetermined individuals in the Atlantic area was half of Cantabrian area (35% and 64% respectively). When considering the total reproductive potential (TRP), as an integration of the relative gonadal size and the sexual maturation level of the mussels, the same geographical pattern was observed, with lower values in Cantabrian mussels, where some sites showed TRP values near zero (Ribadesella or Bilbao) and significantly higher TRP values in Atlantic sites ($t=2.54$, $p<0.05$), where maximum values were observed in interior areas of Galician Rias as Pontevedra-Raxó, with TRP value near 60. Glycogen reserves were also significantly different between areas ($t=4.92$, $p< 0.01$), being almost 60% higher in the Atlantic coast.

Table 4. Student´s test for biological parameters of mussels *(M. galloprovincialis)* from the two geographical areas (Atlantic and Cantabrian coasts). CI_{Shell}: Shell condition Index, CI_{Vol}: Volumetric condition index, ST: Shell thickness, GI: Gill Index, GSI: Gonadosomatic Index, HI: Hepatosomatic Index, RI: Rest tissues index, SMI: sexual madurity index, IND: percentage of indeterminate mussels, TRP: Total reproductive potential, CH/Lip: relationship carbohydrates-Lipids and CH: Carbohydrates for a standard individual of 8ml volume of internal cavity. Min: minimum value, Max: maximum value, R: range, SD: standard desviation, CV: variance coefficient and n: number of sites.

	Atlantic coast							*Cantabrian coast*							*Atl. vs Cant.*	
Variable	Min	Max	R	Mean	SD	CV	n	Min	Max	R	Mean	SD	CV	n	t	p
CI_{Shell}	10.14	14.12	3.98	12.14	1.23	10.16	11	8.77	12.46	3.69	10.40	1.05	10.09	12	3.65	**0.001**
CI_{Vol}	4.67	5.60	0.93	5.12	0.35	6.80	11	3.72	6.21	2.49	4.58	0.63	13.68	12	2.48	**0.021**
ST	0.143	0.180	0.037	0.16	0.01	7.89	11	0.141	0.187	0.046	0.165	0.013	7.79	12	-0.55	0.585
GI	6.99	10.84	3.86	8.77	1.00	11.40	11	6.41	10.84	4.43	9.34	1.21	12.93	12	-1.23	0.231
GSI	6.81	19.46	12.66	11.88	4.21	35.46	11	0.03	15.66	15.62	7.38	4.04	54.72	12	2.61	0.002
HI	11.72	15.16	3.44	13.45	12.20	9.07	11	11.45	14.93	3.48	13.49	0.92	6.85	12	-0.10	0.918
RI	55.79	71.89	16.10	65.91	5.48	8.32	11	63.01	75.77	12.76	69.78	3.33	4.77	12	-2.06	0.051
SMI	0.80	2.93	2.13	1.80	0.73	40.59	11	0.00	2.14	2.14	0.93	0.66	70.72	12	3.02	**0.006**
IND	0.00	80.00	80.00	40.45	0.71	1.76	11	14.00	100.00	86.00	64.42	24.97	38.77	12	-2.35	**0.028**
TRP	0.10	0.61	0.50	0.28	0.18	65.10	11	0.00	0.36	0.36	0.12	0.11	86.84	12	2.54	**0.019**
CH/Lip	1.85	4.55	2.70	2.74	0.71	26.08	11	1.30	2.50	1.21	1.72	0.37	21.70	12	4.33	**0.000**
CH	85.28	139.97	54.69	109.98	17.04	15.50	11	48.65	124.24	75.59	71.29	20.33	28.52	12	4.92	**0.000**

4.2 Pollution

Table 5 shows the concentrations of pollutants accumulated in wild mussels from the N-NW Spanish coast.

With respect to metal pollution, Hg ranged from 0.061 to 0.852 mg kg dw^{-1}, and the most polluted sites were 30, 31 and 32; while Pb ranged from 0.662 to 33.4 mg kg dw^{-1}, and the most polluted sites were 28 and 32. These sites were all located in the Cantabrian area. Cu and Zn ranged from 4.56 and 135 to 9.17 and 367 mg kg dw^{-1}, respectively, being higher also in the Cantabrian area. On the contrary, Cd and As, which ranged from 0.388 and 4.07 to 2.90 and 12.8 mg kg dw^{-1}, respectively, showed higher values in the Atlantic area.

The highest concentrations of PAHs where found in sites 28, 29 and 33 and ranged from 13.25 to 1015 µg kg dw^{-1},. PCBs ranged from 0.831 to 17.2 µg kg ww^{-1} and the highest values were found in sites 10, 19, 33 and 37. BDEs concentrations ranged from 0.057 to 0.790 µg kg ww^{-1}, with the highest values detected in sites 16, 31 and 37. Organochlorinated pesticides, HCH, DDT and chlordanes ranged from 0, 0.077 and 0.022 to 0.078, 1.197 and 0.814, respectively, and the highest values were found in sites 28, 33 and 37 for HCH, 19, 33 and 41 for DDT, and 2, 12, and 31 for chlordanes.

Table 5. Concentrations of pollutants in the soft tissues of mussels (*Mytilus galloprovincialis*) from the N–NW Spanish coast. Metals (mg/kg mussel dry weight), Σ_{12}PAHs: sum of 12 polycyclic aromatic hydrocarbons (µg/kg mussels dry weigth), Σ_9BDEs: sum of 9 polybrominated diphenyl ethers (µg/kg mussels dry weigth), Σ_7PCBs: sum of 7 polychlorinated byphenyls (µg/kg mussels dry weigth), ΣDDTs: sum of p,p'-DDE, 4,4'-DDD and 4,4'-DDT (µg/kg mussels dry weigth), ΣHCHs: sum of α- HCH and γ-HCH, ΣChlordanes: sum of trans-chlordane and cis-chlordane (µg/kg mussels dry weigth), and CPI: Chemical Pollution Index.

Nº	Sampling site	Metals						Σ_{12}PAHs	Σ_9BDEs	Organochlorines				CPI
		Hg	Pb	Cd	Cu	Zn	As			Σ_7PCB	ΣDDTs	ΣHCHs	Σchlordanes	
								Atlantic coast						
2	Oia	0.096	0.99	1.220	5.42	305	7.4	13.3	0.126	1.06	0.08	0.066	0.122	-1.0
3	Samil	0.096	3.15	0.587	5.22	268	12.5	49.6	0.346	6.52	0.43	0.043	0.104	1.2
5	Cabo Home Pontevedra	0.061	1.58	1.260	4.56	275	10.3	35.6	0.084	2.40	0.24	0.019	0.098	-0.9
7	Raxó	0.196	1.69	0.583	6.08	185	9.6	140.6	0.221	2.45	0.48	0.047	0.043	1.1
8	Chazo	0.081	0.66	0.543	6.45	203	6.4	184.6	0.057	1.19	0.35	0.049	0.042	-0.7
12	Punta Insua	0.071	1.58	2.900	5.81	290	12.8	20.5	0.064	0.87	0.12	0.051	0.814	-1.0
14	Corme	0.064	0.80	1.450	5.01	259	7.9	32.4	0.058	1.08	0.10	0.000	0.029	-1.1
16	A Coruña TC	0.098	3.56	0.583	6.45	223	5.3	217.6	0.799	10.22	0.37	0.017	0.040	1.9
19	Ferrol Palma	0.095	1.71	0.444	7.80	243	4.6	130.6	0.348	16.18	1.20	0.039	0.056	1.8
21	Cedeira	0.072	0.67	0.388	6.09	135	4.1	78.7	0.149	3.00	0.29	0.038	0.026	-0.9
22	Espasante	0.075	0.91	0.627	6.23	237	6.2	29.7	0.077	2.11	0.12	0.045	0.039	-1.5
								Cantabrian coast						
24	Ribadeo	0.116	0.84	0.442	6.20	244	4.6	23.0	0.068	1.90	0.34	0.015	0.063	-1.8
26	Luarca	0.137	1.60	0.615	6.85	267	7.4	30.4	0.324	0.83	0.29	0.052	0.099	-0.6
28	Avilés	0.402	20.2	1.200	7.13	296	10.0	1015.2	0.111	3.40	0.08	0.078	0.027	3.4
29	Gijón	0.343	2.83	0.682	7.56	269	9.5	591.8	0.188	3.11	0.18	0.052	0.027	2.4
30	Ridadesella	0.852	2.62	0.722	9.17	173	8.0	63.6	0.122	1.04	0.26	0.015	0.063	0.5
31	SV Barquera	0.438	1.45	0.571	4.92	289	6.0	103.9	0.541	1.70	0.21	0.041	0.105	0.9
32	Suances Santander	0.496	33.40	0.989	5.66	367	6.1	88.3	0.15	4.89	0.17	0.016	0.022	2.1
33	Pantalán Santander	0.196	5.60	0.757	8.45	315	5.9	674.4	0.155	17.17	0.65	0.073	0.041	3.7
34	Pedreña	0.185	2.33	0.514	6.00	263	5.1	123.6	0.149	5.56	0.25	0.025	0.034	0.7
36	Castro Urdiales	0.182	3.33	0.496	8.09	233	6.9	48.7	0.174	4.14	0.16	0.039	0.048	0.5
37	Bilbao Azcorri	0.207	4.99	0.796	8.46	325	8.7	250.2	0.683	15.93	0.31	0.073	0.052	3.6
41	Hondarribia	0.106	3.80	0.516	8.20	212	4.6	62.6	0.373	9.42	0.69	0.049	0.039	1.7

According to Beiras *et al*. (2012) only those chemicals showing loadings >0.7 in the PCA were selected to calculate the CPI, namely: Cu, Zn, Cd, As, Pb, BDEs, PCBs, DDTs, HCHs, chlordanes and B(a)P. Figure 25 shows CPI values calculated for each sampling site, which takes positive values when pollutants exceed on average the environmental quality criteria and negative values otherwise. CPI can be categorized into three groups in order to classify sampling stations attending to their pollution state (Bellas *et al*., 2014): 'low pollution' when CPI≤ 0, 'moderate pollution' when 0 <CPI≤

1 and 'high pollution' when CPI> 1. The highest CPI values were found at stations 28, 29, 32, 33 and 37, with CPI>2, and another five stations (3, 7, 16, 19 and 41) showed CPI values >1, indicating 'high pollution'. Four stations showed CPI values between 0 and 1, indicating 'moderate pollution', and nine stations showed CPI values ≤0, indicating 'low pollution'. CPI also showed differences between coasts (t=-2.41; p<0.05) being the majority of polluted sampling sites located in the Cantabrian coast.

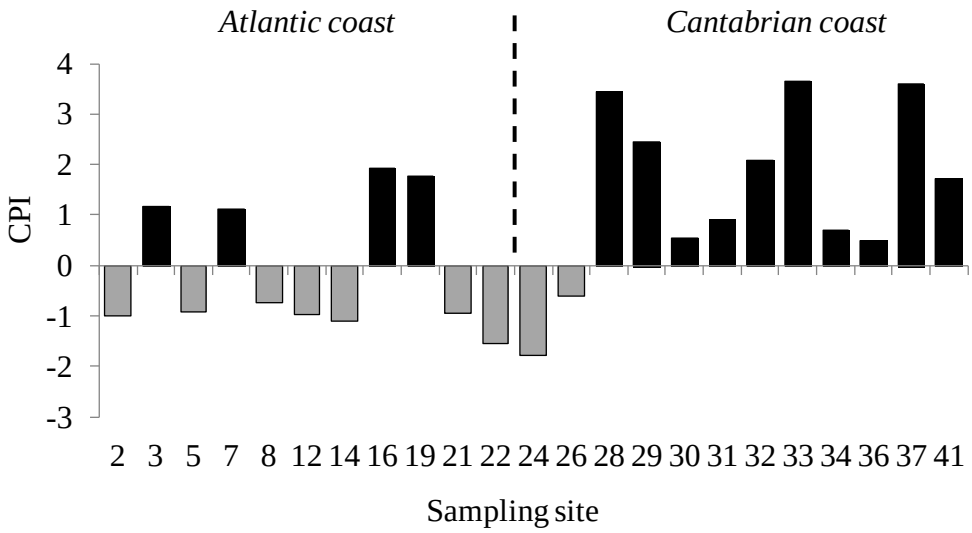


Figure 25. Chemical pollution index (CPI) of the 23 sampling sites from the Spanish Marine Pollution monitoring program (SMP) along the N-NW Spanish coast obtained as the summation of chemical data selected from the CPI calculation (Cu, Zn, Cd, As, Pb, Σ_9BDEs, Σ_7PCBs, ΣDDTs, ΣHCHs, ΣChlordanes and B(a)p).

4.2.1 Pollution vs Biology

The relationship between biological parameters and chemical pollution were considered in this study (Table 6). According to the differences in biological variables showed above, subsequent analyses were conducted in both regions separately. In Atlantic sites, CI_{vol} was correlated positively with Cu, DDTs, PAHs and CPI, and negatively with Zn. GSI showed positive correlations with Hg and PAHs. CH was also positively correlated with Hg. Regarding ST, negative correlations with some organic pollutants were observed, as with BDEs, PCBs or DDTs, and as a consequence with CPI.

Table 6. Pearson correlation coefficients among the different biological and chemical variables tested in the two different coasts (Altantic and Cantabrian coasts). ST: Shell thickness, CI_{vol}: Condition index, GI: Gill Index, GSI: Gonadosomatic Index , HI: Hepatosomatic Index , SMI: Sexual madurity index, CH: Carbohydrates per individual, Σ_{12}PAHs: sum of 12 polycyclic aromatic hydrocarbons, Σ_9BDEs: sum of 9 polybrominated diphenyl ethers, Σ_7PCBs: sum of 7 polychlorinated byphenyls, ΣDDTs: sum of p,p'-DDE, 4,4'-DDD and 4,4'-DDT, ΣHCHs: sum of α- HCH and γ-HCH, Σchlordanes: sum of trans-chlordane and cis-chlordane and CPI: Chemical pollution index.

Pollutant	CPI	Biological parameters						
		ST	CI_{vol}	GI	GSI	HI	SMI	CH
		Atlantic coast						
Cd	-0.424	0.438	-0.453	-0.0925	-0.457	0.149	-0.246	-0.241
Cu	0.483	-0.250	**0.695***	-0.2123	0.146	-0.032	0.300	-0.439
Hg	0.542	0.028	0.514	-0.1036	0.574	0.359	0.389	0.525
Pb	**0.785****	-0.413	0.116	-0.1086	0.030	0.028	-0.132	0.353
Zn	-0.143	0.051	-0.524	0.1216	-0.345	-0.205	-0.141	-0.124
As	-0.075	0.445	-0.379	0.1861	0.003	0.475	-0.003	0.359
Σ_9BDEs	**0.836****	**-0.629***	0.344	-0.2811	0.027	-0.206	-0.152	0.178
Σ_7PCBs	**0.813****	**-0.710***	0.471	-0.1058	-0.014	-0.349	0.070	-0.223
ΣHCHs	-0.096	0.184	-0.047	0.3537	0.061	0.354	0.107	-0.231
Σchlordanes	-0.216	0.406	-0.286	-0.0303	-0.411	0.357	-0.197	-0.338
ΣDDTs	**0.738****	-0.535	**0.678***	0.0789	0.282	-0.079	0.386	-0.145
Σ_{12}PAHs	**0.652***	-0.345	**0.866*****	-0.1104	**0.603***	0.236	0.422	0.301
CPI		-0.571	0.581	-0.1523	0.298	-0.019	0.155	0.259
		Cantabrian coast						
Cd	**0.677***	0.008	-0.403	0.256	-0.393	0.189	-0.322	-0.305
Cu	0.34	-0.507	-0.272	0.194	-0.468	-0.264	-0.377	-0.072
Hg	0.109	-0.343	-0.549	0.489	**-0.611***	0.054	-0.487	**-0.576***
Pb	0.411	0.025	-0.346	0.336	-0.325	0.238	-0.218	-0.33
Zn	0.529	0.458	0.002	0.063	0.03	0.352	-0.012	0.061
As	0.468	0.093	-0.416	0.211	-0.386	-0.112	-0.186	-0.316
Σ_9BDEs	0.249	-0.022	-0.162	0.280	-0.120	-0.377	-0.251	0.042
Σ_7PCBs	**0.676***	-0.154	0.045	0.096	-0.152	-0.035	-0.360	0.281
ΣHCHs	**0.713****	0.133	-0.004	-0.101	0.069	-0.221	-0.032	0.085
Σchlordanes	-0.539	0.196	0.14	-0.223	0.335	0.120	0.386	0.090
ΣDDTs	0.115	-0.329	0.305	-0.220	0.151	-0.202	-0.144	0.336
Σ_{12}PAHs	**0.716****	0.211	0.057	-0.187	0.048	0.012	-0.055	0.002
CPI		-0.156	-0.329	0.370	-0.417	-0.240	-0.552	-0.219

On the contrary, weaker correlations between biological parameters and pollutants were detected in the Cantabrian coast. The strongest relationships were found for Hg which showed negative correlations with CI_{vol}, GSI and CH. ST was weakly correlated with Cu, and SMI showed a negative correlation with CPI.

4.3 Biochemical biomarkers

Biomarker results obtained in this study are shown in Table 7 and indicate a wide range of variation between sites. CAT values ranged from 35.51 to 104.08 µmol min^{-1} mg $prot^{-1}$ and presented the highest values in stations 3, 5, 12, 19 and 37. GST and GR ranged from 47.99 to 142.07 and from 15.46 to 45.53 nmol min^{-1} mg $prot^{-1}$ respectively. GST presented the highest values in stations 3, 12, 16, 21, and 29, while GR only presented extreme values in two sites, 29 and 30. LPO showed a strong range of variation between stations, from 0.61 to 1.49 nmol MDA mg $prot^{-1}$, with highest values measured in sites 21, 22, 24, 28 and 41. In general, antioxidant activities were positively correlated between them and inversely correlated to LPO (data not shown), although these relationships were more evident in Cantabrian sites.

Table 7. Biochemical variables (mean and standard deviations) of mussels (*M. galloprovincialis*) from the N-NW Spanish coast collected in November 2012. CAT: Catalase, GST: Glutathione- S-transferase, GR: Glutathione Reductase and LPO: Lipid Peroxidation.

Nº	Sampling site	CAT µmol min^{-1}mg $prot^{-1}$	GST nmol min^{-1}mg $prot^{-1}$	GR nmol min^{-1}mg $prot^{-1}$	LPO nmolMDA mg $prot^{-1}$
		Atlantic coast			
2	Oia	56.2±14.5	101.3±17.36	21.9±9.0	0.84±0.26
3	Samil	104.1±24.1	124.0±28.6	27.3±8.1	0.91±0.18
5	Cabo Home	96.4±12.7	80.9±20.3	29.4±7.0	0.67±0.27
7	Pontevedra Raxó	76.1±14.8	88.2±10.2	15.5±5.9	0.62±0.25
8	Chazo	58.7±12.2	103.2±7.7	16.1±1.2	0.64±0.27
12	Punta Insua	88.9±10.1	142.1±38.8	31.5±8.6	0.64±0.03
14	Corme	68.6±19.1	104.2±18.7	20.7±4.8	0.88±0.32
16	A Coruña TC	71.6±27.9	135.1±22.3	24.2±6.9	0.86±0.32
19	Ferrol Palma	89.8±30.3	100.0±23.9	21.7±5.4	0.62±0.18
21	Cedeira	67.5±10.4	121.0±32.1	20.9±3.0	1.01±0.26
22	Espasante	70.5±12.1	96.2±25.3	22.7±4.2	1.49±0.51
		Cantabrian coast			
24	Ribadeo	35.5±11.8	48.0±24.1	21.2±6.6	1.44±0.58
26	Luarca	67.8±15.7	106.0±42.1	27.8±9.4	0.73±0.27
28	Avilés	56.7±17.3	96.7±36.6	29.7±13.1	0.97±0.34
29	Gijón	62.9±11.8	129.1±19.7	45.5±8.9	0.72±0.21
30	Ridadesella	88.4±33.3	90.1±31.4	37.6±9.3	0.81±0.26
31	SV Barquera	56.5±13.5	64.0±16.0	23.1±6.3	0.81±0.19
32	Suances	45.8±15.5	58.8±21.5	28.1±15.0	0.89±0.45
33	Santander Pantalán	65.2±4.0	97.6±22.8	21.0±4.9	0.94±0.36
34	Santander Pedreña	46.6±12.8	96.6±16.8	24.5±9.1	0.76±0.17
36	Castro Urdiales	50.1±17.7	99.5±18.7	28.8±9.2	0.77±0.23
37	Bilbao Azcorri	101.1±5.9	106.2±25.3	30.2±6.4	0.61±0.42
41	Hondarribia	60.6±30.6	101.0±22.2	26.0±7.0	1.04±0.31

4.3.1. Biomarkers vs. Biology

Correlations between biological indices and biomarkers are shown in Table 8. In the Atlantic coast, GR showed a negative correlation with CI_{vol} and GSI. GST showed a significant negative correlation with SMI and also a weak negative correlation with GSI.

Biochemical biomarkers showed a similar pattern in the Cantabrian coast, where CAT was negatively correlated with GSI, SMI and with less strength with CI_{vol}. GR also showed a negative correlation with GSI. GST showed a negative correlation with HI. Contrary to antioxidant activities, LPO showed strong positive correlations with CI_{vol}, GSI and CH, and also a negative correlation with GI.

In summary, it seems that antioxidant activities presented higher values in mussels with poorer nutritive state, considered this as CI, SMI or GSI, although this pattern seems to be more evident in Cantabrian mussels. On the contrary, LPO was clearly positively related to mussel condition (CI, GSI, CH) in a way that higher LPO was observed in mussels with higher condition.

4.3.2. Biomarkers vs. Pollution

Correlations between biomarkers and pollutants are shown in Table 8. Significant positive correlations were observed in the Atlantic coast between CAT and metals as As and Pb. GR showed also positive correlations with Cd, Zn, As and chlordanes, whereas a weak negative correlation was found with Hg.

In the Cantabrian coast, CAT was positive correlated with Cu, BDEs and As; GST was positively correlated with Cu, HCHs and As; GR showed a strong positive correlation with As ($r=0.728$, $p<0.01$); and LPO showed a weak negative correlation with As.

Table 8. Pearson correlation coefficients among the different biochemical biomarkers in the two different coast studied (Atlantic and Cantabrian coasts) and biological/ chemical. CAT: Catalase (µmol/min/mg prot), GST: Glutation S-transferase (nmol/min/mg prot), GR: Glutation Reductase (nmol/min/mg prot) and LPO: Lipid Peroxidation (nmol/MDA/mg prot), ST: Shell thickness, CIvol: Condition index, GI: Gill Index, GSI: Gonadosomatic Index , HI: Hepatosomatic Index , SMI: Sexual madurity index, CH: Carbohydrates per individual, Σ12PAHs: sum of 12 polycyclic aromatic hydrocarbons, Σ9BDEs: sum of 9 polybrominated diphenyl ethers, Σ7PCBs: sum of 7 polychlorinated byphenyls, ΣDDTs: sum of p,p'-DDE, 4,4'-DDD and 4,4'-DDT, ΣHCHs: sum of α- HCH and γ-HCH, Σchlordanes: sum of trans-chlordane and cis-chlordane and CPI: Chemical pollution index.

	Biological parameters							Chemical data											
	ST	CI_{vol}	GI	GSI	HI	SMI	CH	Cd	Cu	Hg	Pb	Zn	As	Σ_9BDEs	Σ_7PCB	ΣHCHs	Σchlordanes	ΣDDTs	Σ_{12}PAHs	CPI
								Atlantic coast											
CAT	-0.111	-0.083	0.266	-0.066	0.187	0.055	0.130	0.177	-0.153	-0.031	0.542	0.309	**0.605***	0.154	0.372	-0.163	0.298	0.360	-0.208	0.374
GST	-0.044	-0.195	-0.424	-0.517	-0.017	**-0.629***	-0.344	0.329	0.128	-0.231	0.446	0.018	0.128	0.423	0.149	0.030	0.538	-0.114	0.111	0.197
GR	-0.033	-0.576	0.197	**-0.585***	-0.021	-0.395	-0.202	**0.613***	-0.391	-0.518	0.387	**0.608***	0.557	0.056	0.036	-0.114	**0.630***	-0.214	-0.499	-0.102
LPO	0.245	-0.465	-0.286	-0.383	-0.337	-0.345	-0.232	-0.254	-0.063	-0.286	-0.131	-0.110	-0.279	-0.054	-0.159	-0.023	-0.274	-0.384	-0.323	-0.361
								Cantabrian coast											
CAT	-0.407	-0.490	0.411	**-0.616***	-0.222	**-0.591***	-0.168	0.217	**0.659***	0.309	-0.211	-0.072	0.523	0.562	0.400	0.414	0.161	0.131	0.084	0.427
GST	-0.298	-0.359	0.227	-0.327	-0.562	-0.298	-0.298	0.058	**0.593***	-0.158	-0.293	-0.161	0.554	0.153	0.261	**0.584***	-0.239	0.081	0.383	0.435
GR	-0.181	-0.452	0.359	-0.544	-0.425	-0.389	-0.451	0.226	0.392	0.481	0.005	-0.224	**0.727****	-0.075	-0.267	0.022	-0.265	-0.401	0.216	0.210
LPO	0.297	**0.738****	**-0.698***	**0.612***	0.329	0.461	**0.625***	-0.152	-0.205	-0.211	0.051	-0.173	-0.510	-0.471	-0.147	-0.298	-0.070	0.301	-0.031	-0.393

* Correlations are significant at 0.05 level

** Correlations are significant at 0.01 level

***Correlations are significant at 0.001 level

4.4 Physiological biomarkers

Mean SFG values and their standard deviations are shown in Table 9, together with all the physiological rates used in the SFG estimation. SFG ranged from 7.02 to 31.02 J g^{-1} h^{-1} with a variance coefficient of 32.6%. Maximum values of SFG were found in stations 3 and 37, whereas the minimum values were found in stations 8, 21 and 24. CR was expressed in relation to total meat weight, length and gill size. $CR_{W\text{-}st}$ ranged from 1.96 to 4.25 L h^{-1}, for a specimen of 1 g dry weight. When CR was length-standardized ($CR_{L\text{-}st}$) the range of CR values was lower (2.49-4.29 L h^{-1} for a specimen of 60 mm) than in the case of the weight standardization, indicating that length standardization reduces CR variability between sites. In the present survey, we observed a higher AE range than in previous surveys, from 38.5 % at site 21 to almost double (79.1%) at site 3. RR ranged from 4.1 to 7.6 J g^{-1} h^{-1}.

It is worth highlighting that a different pattern was observed in the physiological parameters between areas. In this connection, SFG values measured in Atlantic mussels were mainly dependent on AE ($r=0.928$, $p<0.01$) and to a lesser extent on RR ($r=-0.712$, $p<0.05$), whereas SFG variability in the Cantabrian coast was positively associated to CR, independently of the standardization applied ($p<0.01$), and secondly and to a lesser extent with AE. In Cantabrian sites, SFG values were not related to RR ($p>0.05$).

4.4.1. Biomarkers vs. Biology

Only a positive but weak correlation was detected between $CR_{L\text{-}st}$ and CI ($r= 0.533$, $p=0.09$) in the Atlantic coast (Table 10). On the contrary, highly significant relationships between physiological rates and biological parameters were found in the Cantabrian coast. Independently of the standardization method used, CR was higher in mussels with lower condition, measured as CI_{vol}, GSI or SMI, although these relations were stronger for $CR_{w\text{-}st}$. A positive correlation with GI was also evident. RR was negatively correlated with condition, although almost significance was only achieved for SMI ($r= -0.538$, $p=0.07$). On the other hand, no significant correlations were detected between AE and biological variables. As a result, SFG was negatively related to mussel condition (with CI_{vol} $r= -0.683$, $p<0.05$, with GSI $r= -0.667$, $p<0.05$, or with SMI $r= -0.489$, $p=0.10$) and positively with GI. Another relevant result was the inverse relationship observed between RR and ST, indicating that higher RR were observed in mussels with lower ST.

Table 9. Physiological parameters (mean and standard deviations) of mussels collected through the 23 sampling sites of the N-NW Spanish Coast under standardized laboratory conditions (15ºC, filtered seawater 1µm, 0.55 mg/L of algal cells). $CR_{L\text{-}st}$: Clearance rate (60 mm length), $CR_{W\text{-}st}$: Clearance rate (1g dry weight), AR: absorption rate, AE: absorption efficiency, RR: respiration rate and SFG: Scope for Growth.

Nº	Sampling site	Physiological parameters						
		$CR_{L\text{-}st}$ L/h	$CR_{W\text{-}st}$ L/h	IR J/g/h	AE %	AR J/g/h	RR J/g/h	SFG J/g/h
				Atlantic coast				
2	Oia	2.9±0.6	3.2±0.8	40.8±10.2	73.3±10.6	30.0±8.5	4.4±0.9	25.6±8.1
3	Samil	3.4±0.7	3.5±0.8	45.0±10.1	79.1±4.1	35.8±8.6	4.9±0.7	30.8±8.4
5	Cabo Home	3.6±0.6	3.9±0.6	49.7±8.4	60.4±10.8	30.3±8.7	4.1±0.8	26.3±8.7
7	Pontevedra Raxó	4.2±1.0	3.7±0.8	48.0±9.8	67.2±5.9	32.1±6.8	5.7±1.1	26.4±6.6
8	Arosa	3.2±0.5	3.0±0.6	37.9±7.4	48.1±12.3	18.2±6.2	7.2±0.8	11.0±6.0
12	Punta Insua	3.5±0.3	3.9±0.5	49.7±5.9	58.3±9.8	28.9±5.5	6.2±0.9	22.7±5.4
14	Corme	3.6±0.6	3.8±0.6	48.3±7.9	44.1±10.1	21.3±6.2	4.9±0.5	16.3±6.1
16	A Coruña TC	3.6±0.5	3.7±0.7	46.9±8.8	63.2±6.1	29.9±7.0	5.1±0.7	24.8±6.7
19	Ferrol Palma	3.6±0.5	3.5±0.6	44.8±7.7	62.0±17.6	27.5±8.7	6.2±1.0	21.3±8.7
21	Cedeira	3.6±0.5	3.5±0.6	45.0±8.0	38.5±7.1	17.6±5.0	6.7±0.9	10.8±4.7
22	Espasante	3.2±0.4	3.3±0.4	42.7±5.2	45.9±13.4	19.7±6.3	6.0±0.8	13.7±6.3
				Cantabrian coast				
24	Ribadeo	2.4±0.8	2.0±0.6	24.8±7.4	52.7±9.9	12.9±4.0	5.8±0.6	7.0±4.0
26	Luarca	3.5±0.7	3.6±0.7	45.9±9.2	62.3±10.4	28.7±7.6	6.0±0.9	22.6±7.7
28	Avilés	3.1±0.5	3.2±0.4	41.7±5.7	65.6±6.2	27.3±4.5	5.7±0.7	21.6±4.6
29	Gijón	3.2±0.4	3.5±0.6	44.8±7.9	73.5±12.0	32.6±6.4	6.8±1.3	25.8±6.4
30	Ridadesella	3.2±0.6	3.8±0.6	48.3±8.4	58.7±13.9	28.5±9.4	7.6±1.1	20.9±9.7
31	S Vicente	3.2±0.9	3.1±0.6	38.8±8.0	53.2±10.1	20.7±5.62	6.8±0.6	13.8±5.8
32	Suances	3.4±0.6	4.0±0.7	50.2±8.6	51.8±0.00	26.1±4.4	6.6±1.0	19.4±4.2
33	Santander Pantalán	3.2±0.6	3.0±0.6	38.1±8.0	50.3±11.27	19.0±5.3	6.9±0.8	12.0±5.4
34	Santander Pedreña	3.1±0.7	3.5±0.8	44.1±9.8	60.4±8.5	26.3±5.9	7.4±1.5	18.9±5.9
36	Castro Urdiales	3.3±0.7	3.7±0.7	47.5±8.8	56.4±10.4	26.7±6.7	6.7±1.0	20.1±6.5
37	Bilbao Azcorri	3.5±0.7	4.2±0.8	53.8±10.3	69.1±13.2	36.9±9.0	5.8±0.8	31.0±9.0
41	Hondarribia	2.9±0.5	3.0±0.5	38.5±6.7	59.8±11.6	23.3±7.2	7.3±0.8	15.9±7.5

Table10. Pearson correlation coefficients among the physiological parameters considered and biological/chemical variables tested in the two different coast (Altantic and Cantabrian coasts). CR_{W-st} : Clearance rate (1g dry weight), CR_{L-st}: Clearance rate (60 mm length), AR: absorption rate, AE: absorption efficiency, RR: respiration rate, SFG: scope for growth, ST: Shell thickness, CI_{vol}: Condition index, GI: Gill Index, GSI: Gonadosomatic Index , HI: Hepatosomatic Index , SMI: Sexual madurity index, Σ_{12}PAHs: sum of 12 polycyclic aromatic hydrocarbons, Σ_9BDEs: sum of 9 polybrominated diphenyl ethers, Σ_7PCBs: sum of 7 polychlorinated byphenyls, ΣDDTs: sum of p,p'-DDE, 4,4'-DDD and 4,4'-DDT, ΣHCHs: sum of α- HCH and γ-HCH, Σchlordanes: sum of trans-chlordane and cis-chlordane and CPI:Chemical pollution index.

	Biological parameters						Chemical data												
	ST	CI_{vol}	GI	GSI	HI	SMI	Cd	Cu	Hg	Pb	Zn	As	Σ_9BDEs	Σ_7PCBs	ΣHCHs	Σchlordanes	ΣDDTs	Σ_{12}PAHs	CPI
										Atlantic coast									
CR_{W-st}	0.021	-0.117	-0.201	-0.243	0.031	-0.167	0.469	-0.344	0.059	0.332	0.142	0.461	0.130	0.046	-0.540	0.369	-0.040	-0.220	0.151
CR_{L-st}	-0.001	0.532	-0.206	0.361	0.309	0.312	-0.086	0.118	**0.590***	0.277	-0.427	0.129	0.257	0.222	-0.380	-0.040	0.355	0.348	0.482
AE	-0.319	-0.059	0.254	0.127	0.077	0.017	0.025	-0.121	0.454	**0.658***	0.516	0.500	0.400	0.291	0.337	0.112	0.232	-0.010	0.564
AR	-0.276	-0.072	0.186	0.066	0.123	-0.013	0.165	-0.227	0.443	**0.734***	0.498	**0.620***	0.424	0.28	0.122	0.223	0.197	-0.05	0.577
RR	0.303	0.486	-0.138	0.190	0.374	0.248	-0.174	**0.654***	0.022	-0.374	**-0.633***	-0.368	-0.180	0.029	0.303	0.107	0.257	0.391	-0.060
SFG	-0.293	-0.134	0.187	0.032	0.057	-0.048	0.174	-0.299	0.397	**0.717***	0.541	**0.613***	0.408	0.249	0.067	0.186	0.141	-0.100	0.531
										Cantabrian coast									
CR_{W-st}	-0.423	**-0.894*****	**0.852*****	**-0.845*****	-0.218	**-0.593***	0.376	0.297	0.349	0.295	0.227	0.527	0.322	0.150	0.147	-0.170	-0.360	-0.030	0.425
CR_{L-st}	-0.205	**-0.758****	**0.671***	**-0.603***	-0.116	-0.383	0.352	0.114	0.219	0.237	0.410	0.476	0.453	0.167	0.300	0.102	-0.280	0.009	0.405
AE	-0.046	-0.268	0.149	-0.291	-0.491	-0.292	0.208	0.306	-0.028	-0.148	-0.066	**0.731****	0.260	-0.010	0.437	-0.220	-0.290	0.356	0.321
AR	-0.301	**-0.725****	**0.640***	**-0.715****	-0.384	-0.548	0.348	0.375	0.213	0.100	0.118	**0.726****	0.384	0.119	0.332	-0.200	-0.370	0.148	0.448
RR	**-0.725****	-0.351	0.477	-0.413	-0.427	-0.538	-0.335	0.199	0.345	-0.207	-0.443	-0.354	-0.150	0.016	-0.380	-0.130	0.327	-0.270	-0.010
SFG	-0.225	**-0.683***	**0.585***	**-0.667***	-0.337	-0.489	0.379	0.351	0.176	0.120	0.162	**0.755****	0.395	0.116	0.368	-0.180	-0.400	0.174	0.440

* Correlations are significant at 0.05 level

** Correlations are significant at 0.01 level

***Correlations are significant at 0.001 level

4.4.2. Biomarkers vs. Pollution

As well as with biochemical biomarkers, chemical pollution was associated with physiological biomarkers (Table 10). In the Atlantic coast, $CR_{w\text{-}st}$ did not show any correlation with pollution, however $CR_{L\text{-}st}$ was positively correlated with Hg. AE showed positive correlations with Pb, Zn, and with CPI. RR showed a positive correlation with Cu and a negative correlation with Zn. Finally, SFG showed positive correlations with metals as Pb, Zn or As.

In the Cantabrian coast, only As showed correlations with physiological biomarkers in this area. Positive correlations were found between As and $CR_{w\text{-}st}$ ($r=0.527$, $p=0.07$) and AE and as result, also with SFG.

5. Discussion

5.1 Biological variability

The applicability of biomarkers on marine pollution monitoring programs is conditioned by the ability to discriminate between mussel pollutant-responses from the influence of variability in natural processes (Thain *et al*., 2008). The larger the area covered by a monitoring program, the higher the natural variability will be expected on environmental factors such as food availability, temperature or salinity, and therefore on intrinsic factors such as age, condition or reproductive state. In the present study, we have characterized the wide biological variability in mussel populations used for the assessment of pollution along an extensive area included within the Spanish Marine Pollution monitoring program (SMP). This is the first time that such an exhaustive quantitative and qualitative biological characterization is carried out within the SMP.

The highest variability was observed on the mantle relative size (GSI), which is closely related to mussel condition, the generalist index indicative of mussel physiological state. Mantle tissues serve to both accumulation of reserves and gonad development in *Mytilus* sp. (Gabbott and Peek, 1991). In this sense, a general annual reproductive/reserves cycle has been described according to which gametogenesis takes place between late autumn and winter until the beginning of spring, when the gonad is completely ripe and spawning occurs (Seed, 1976; Bayne, 1976, 1984; Lubet, 1959). However, this general pattern widely differs within the same population depending on latitude and food availability, and also between years. On the other hand, the cyclical nature of gametogenesis in *Mytilus* is less evident in populations located in low-latitudes (Gabbott, 1975), as the area used for this study, which comprises the Galician coastline and the Bay of Biscay. In the Galician Atlantic coast, for instance, two reproductive cycles have been described, with an important spawning peak in spring and a second and less intense peak in autumn (September-October) (Cáceres-Martínez and Figueras, 1998; 2007). As a consequence, in these latitudes it is more difficult to observe a well defined resting period between these two spawning episodes (Villalba, 1995). In addition, Bayne (1976) distinguished between “conservative species” which show one cycle of reserves storage in summer, gametogenesis in autum-winter and

spawning episodes in spring, from "opportunistic species" whose gametogenesis takes place whenever food available conditions were optimal. *Mytilus sp*. behaves as a "conservative species" in situations of low food availability and as an "opportunistic species" when trophic conditions are favorable (Rodhouse *et al*, 1984). Considering that the present study was carried out in November, mussels at the beginning of gametogenesis could be expected. However, the results obtained evidenced that the 23 mussel populations differ both in their gonadal development (GSI from 0 to 20) and in their gonadal maturity (SMI averages values from 0 to 3 considering an scale from 0 to 4). In fact, mussels with high mantle tissue development (GSI values near 20% of total dry body tissues), also presented advanced gametogenetic stages (SMI, stages 3 or 4), evidenced by a strong positive correlation between both variables (Figure 24). This finding is in agreement with the "opportunistic strategy" of *Mytilus spp.* populations, according to the classification of Bayne (1976), in such a way that, if environmental conditions are favorable, mussels would experience important gonadal development. The maximum GSI values reported here are similar to the maximum values observed for this species along the annual cycle (Sukhotin and Flyachinskaya, 2009; Schmidt *et al*., 2013; Suarez *et al*., 2013). Therefore, the variability of gonadal development in the study area, which covers a coastline longer than 2500 km, is higher than the expected variability in one site throughout a year, i.e. all gametogenetic stages observed during the year can coexist at the same season (November) when different sites in the sampling area are studied.

Regarding the biochemical reserves (storage reserves), as mentioned before, the reproductive cycle in bivalves is closely related to the cycle of storage and utilization of glycogen (Gabbott 1976). In fact, it has been widely described that energy components stored mainly in the mantle during periods of sexual rest and food abundance are used as fuels for subsequent gametogenesis (Gabbott and Whittle, 1986). Data presented here show a wide range of carbohydrates content between sites, from values of less than 20 until 40 % of the total organic matter. These values are included within the levels described for this season (autumn) in areas from the Galician Atlantic coast, and also within the levels registered in spring-summer where food availability is higher, being substantially higher than the values observed during maximal gonadal development (late winter and spring) (Freites *et al*, 2003). In other latitudes, maximum values (35% of total dry weight) have been described in spring and summer, medium values in autumn (20%) and minimum values previous to spring spawning (10%) (de Zwaan and Zandee, 1972). In this study, glycogen variability observed between the 23 sampling sites seem to be lower than the gonadal development variability (GSI, see above), since values observed are not as low as minimum values described in the literature.

A clear geographical pattern was observed here both for the gonadal development (Figure 24) and for the carbohydrates levels (Table 3). Atlantic populations show higher values of energetic reserves and also a highly differentiated gonadal development, in contrast to Cantabrian populations. Both, gonadal development and reserve levels, are closely linked to food availability (Bayne, 1976; Gabbott, 1976),

evidencing that Atlantic habitats are more productive than the Cantabrian ones. Spatial differences in gonadal development in bivalves between sampling sites, even between close areas, and its association with food availability, has been reported by several authors (Cáceres-Martínez and Figueras 1998; Villalba, 1995). The Atlantic area presents several upwelling processes that can affect primary production providing an increase in the availability of food (Fraga, 1981), in contrast to the Cantabrian area. It is evident that natural variation of food supply can alter the individual´s capacity to assimilate nutrients, modifying the nutrient storage cycle and also the gametogenic cycle (Newell *et al*., 1982). Although other factors may be involved in the gametogenic cycle (e.g. temperature), Griffiths (1977) concluded that food availability may be of greater importance in maturation of the gonad than temperature. This statement supports the argument suggested by Bayne (1976) that gametogenesis is regulated by nutrient environmental changes.

Differences in mussel nutritive condition found in this study are in agreement with the profusely described environmental characteristics of the North-Atlantic Spanish coastline, according to which two different areas are considered: the Atlantic and the Cantabrian coasts. The Atlantic coast is more productive than the Cantabrian area, because the former is considered as the northern limit of the upwelling system associated to the Eastern North Atlantic Central Water which supplies nutrients to surface waters leading to an increase of primary productivity in these waters (Alvárez *et al*., 2011). Within the Atlantic coast, in the so called "Rías Baixas" (South Galicia) the upwelling phenomenon takes place with higher intensity (Gómez-Gesteira *et al*., 2011) generating highly productive ecosystems. It is in this area where mussels show the highest nutritive condition and reproductive potential: Pontevedra-Raxó, Arousa, Cabo Home. Although main periods of coastal upwelling are occurring in spring and summer (Fraga, 1981), it can frequently be also observed in autumn (de Castro *et al*., 2008) when the present study was carried out. The upwelling phenomenon can also be observed in the Cantabrian coast between late spring and summer, but those events are less intense and continuous than in the Atlantic coast (Gil, 2008; Lavín *et al*., 1998; Llope *et al*., 2003).

5.2 Pollution

A clear difference in the levels of pollutants was observed between the two studied areas, as revealed by the CPI index (Figure 25). In general, the Atlantic coast showed less pollution levels, although some stations as Vigo (site 3), Pontevedra-Raxó (site 7), A Coruña (site 16) and Ferrol (site 19) presented medium values of pollution due to the accumulation of industrial and urban activities, as reported elsewhere (Bellas *et al*., 2014 and references therein). The most polluted sites (CPI>2) are located in the Cantabrian area: Avilés (site 28) presented high values of Pb, PAHs and HCHs, Gijón (site 29) showed high values of Hg and PAHs, Suances (site 32) showed high values of Hg, Pb and PCBs, Santader-Pantalán (site 33) presented high concentrations of PAHs, PCBs, and DDTs, and Bilbao (site 37) presented high concentrations of PAHs, PCBs, BDEs and HCHs. Pollution levels in areas nearby the cities of Avilés, Gijón, Santander

and Bilbao, can be attributed to different industrial and port activities such as iron and steel industry, shipbuilding, chemical and pharmaceutical industry and wood processing, which result in water quality degradation. Suances, though, is a small fishing town (population ca. 8.500 people) located in the Cantabrian area, which showed high metals and PCBs levels that may be caused by the residues of nearby abandoned Hg and Zn-Pb mines and by the proximity of an important industrial area (Irabien *et al*., 2008).

Special attention may be paid to the spatial distribution of Cd and As. Unlike the other metals studied here, maximum values were found in presumably unpolluted sites from the Galician coast, far from urban or industrial areas. Cd showed the same trend in past years according to Bellas *et al*. (2014). In fact, Cd concentrations in mussel tissues are directly related to the intensity of coastal upwelling (Segovia-Zavala *et al*., 2003), which is specially intense and abundant along this area (Álvarez *et al*., 2011; Cabanas, 2000). Arsenic might have a geological origin from local lithology (arsenopyrites), and is transported from rivers to coastal zones according with Costas *et al*. (2011). The Ria circulation strengthened by upwelling enhances the exportation of As to the ocean, affecting the most external sampling sites. Other studies also evidenced higher concentrations of As in clean sites from USA, related with phosphate deposits (Valette-Silver *et al*., 1999). In the same area as in the present study, Besada *et al*. (2014) reported As concentrations fairly homogeneous over all sampling sites, pointed out the difficulty to link As levels in mussel tissues and pollution.

It is noteworthy the inverse relationship between the accumulation of certain organic pollutants such as PCBs, DDTs or BDEs with the shell thickness (ST). If we consider that ST is indicative of age (Frew *et al*., 1989), the accumulation of these organic molecules would be higher in younger individuals, which could be related to its higher activity compared with older individuals (Yap *et al*., 2003). In a similar way, it has been described that animal size clearly influences bioaccumulation of chemical compounds in bivalves where higher concentrations was observed in smaller individuals (Richir and Gobert, 2014). Mechanisms of such size-related bioaccumulation patterns have been be related to variations of physiological processes, as feeding, with size and age (Zhong *et al*., 2013). On another hand, this finding may be also related to the alteration produced by these contaminants on the normal process of shell calcification (Lionetto *et al*, 2012; Zuykov *et al*., 2013)

5.3 Biochemical biomarkers

All the antioxidant activities measured were positively correlated between them and inversely related to lipid peroxidation as a consequence of the capacity of the antioxidant defence system to protect against the effects of ROS production. Similarly, Fernández *et al*. (2010a) described an inverse relationship between antioxidant activities and LPO in mussel gills or Viarengo *et al*. (1991) in mussel glands.

High correlations were found between biochemical biomarkers and biological indices. According to our results, antioxidant activities present higher values in mussels

with a poorer condition, considered this as CI, SMI or GSI, whereas LPO was positively related to mussel condition (CI, GSI, CH). These patterns were more evident in Cantabrian mussels. These results are in agreement with previous studies which showed the effect of environmental (food availability, temperature) and intrinsic (reproduction) factors, on biomarkers levels (Lam, 2009; Van der Oost, 2003). Mussel condition indices used in this study are related to the amount of meat occupying the intervalvar cavity, to the glycogen content in whole mussel tissues and to gonadal development of mussels. Furthermore, all these indices are closely related themselves as it has been explained in the above-sections and they seem to reflect the particular environmental conditions regarding food availability from each site. Therefore, the relationship between antioxidant activities and mussel condition might be explained by both the nutritional state and the gonadal development of mussels, with the added difficulty that both processes are closely related.

Oxidative stress is a highly seasonal process in mussels (Sheehan and Power, 1999) with higher levels of antioxidant activities observed in spring-summer than in autumn-winter (Leiniö and Lehtonen, 2005; Power and Sheehan, 1996; Schmidt *et al*., 2013; Viarengo *et al*., 1991). In these studies, higher activities in spring were associated to higher metabolic states of the animals for this season which is related to the gonad ripening and higher food availability. However, we found higher antioxidant activities in mussels with lower sexual maturity index. An alternative explanation to the higher antioxidant activities observed in warmer months might be related to the influence of temperature on these enzymes. Warmer temperatures increase metabolic rate at mitochondria and the associated increase in ROS production stimulates all the mechanisms of defence against oxidative stress as antioxidant enzymes (Pörtner, 2002). In this sense, Borkovic *et al*. (2005) detected an increase of CAT activity in mussels from the Adriatic Sea collected in May, and suggested that this increase was linked to the increased metabolic activity related to seasonal temperature elevations and intense reproductive activity that occurs in spring. In our study, the temperature did not affect antioxidant activities as sampling period was the same for all sites, and important differences between the sampling sites were not recorded (data not shown).

Contrary to the above mentioned studies, other works have reported higher antioxidant activities, as SOD, GR, GSH-Px or GST, in winter which might be caused by a higher sensitivity to pollutants in the winter season (Borkovic *et al*., 2005), as described in the present thesis. Beyond temperature or sexual maturation, winter months are characterized by low food availability which could explain the higher antioxidant activities observed in our study as it was also pointed out by Borkovic *et al*. (2005). Inverse relationships between antioxidant activities and organism nutritive state were also described in limpets (Ansaldo *et al*., 2007) and fish (Martínez-Alvarez *et al*., 2005; Morales *et al*., 2004; Pascual *et al*., 2003). Despite that low food availability decreases metabolic activities; it also causes degradation of endogenous biochemical components as proteins, glycogen and lipids in order to obtain the energy required by metabolism maintenance (Ansaldo *et al*., 2007). Under this conditions of dietary restriction, tissue

autophagy is induced by the increase of the number and activity of lysosomes (Moore, 2004; Moore *et al*., 2007) where cytoplasmic proteins and organelles are catabolized to generate intracellular nutrients to maintain energy production and biosynthesis processes (Levine, 2005). Lysosomes from digestive glands are sites of ROS generation (Moore *et al*., 2007) and, as a consequence, antioxidant defence systems, as the antioxidant enzymes could be activated. Moreover, an increase of enzymatic activity of the intermediary metabolism has been described to be an indicative of aerobic metabolism under food restriction conditions (Morales *et al*., 2004). For this reason, it has been postulated that animals living in fluctuating environments, as mussels, where autophagy is repeatedly stimulated by natural stressors as food deprivation, will be generically more tolerant of pollutant stress, due to the induced antioxidant defence systems by natural food limitation (Moore *et al*., 2006).

The present study also reports a positive correlation of the LPO with nutritional state (CI, GSI or CH) in mussels from the Cantabrian coast, suggesting that higher nutritional state may be associated with higher reproductive efforts and consequently, with higher metabolic costs, with their associated ROS generation (Bayne, 1976; Dahlhoff *et al*., 2002). On the contrary, a negative correlation is reported here between LPO and GI which should be due to the lower relative gill size in mussels with the highest nutritive condition, as GI and CI or GSI are inversely related ($r=-0.668$, $p<0.001$ and $r=-0.425$, $p<0.05$, respectively). As a complementary explanation, GI has also been negatively associated to ST ($r=-0.409$, $p=0.05$). Considering ST as indicative of age, as pointed out before, it might be concluded that older mussels have lower GI and, from the inverse relation between GI and LPO, higher oxidative stress measured as LPO. Previously, Bellas *et al*. (2014) or Canesi and Viarengo (1997) also related ROS production with age.

The results of the present study showed that the bioaccumulation of different pollutants, apart from biological variables, increase the levels of the antioxidant enzyme activities. It is well know that exposure of aquatic organisms to pollutants may increase ROS generation, which can lead to an imbalance in antioxidant defences, enhance oxidative stress and generate LPO (Fernández *et al*., 2012; Oliva *et al*., 2012; Vlahogianni and Valavanidis., 2007). The correlation analyses conducted here, point to metals as the main cause of the observed effects on biochemical biomarkers, in particular Cu, Zn, Cd and As. The relationship between GR and metals could be attributed to a coordinated regulation of these enzymes, aimed at restoring the GSH pool to permit an efficient antioxidant response as was previously described by Fernández *et al*. (2010a) and Borković-Mitić *et al*. 2013.

Overall, the low or lack of correlation found in this work between the antioxidant biomarkers and some organic contaminants, which are able to enhance the formation of ROS (PCBs, PAHs, DDTs etc.), could be explained by the fact that the oxidative stress biomarkers constitute a global response mechanism to the increased number of pollutants, which are being taken up by the organism and may perturb free radical processes. However, our results agree with previous studies on the same

sampling area (Albentosa *et al*., 2012a; Bellas *et al*., 2014), where organochlorines such as chlordanes in the Atlantic coast, and HCHs in the Cantabrian coast, also showed a significant effect on GR and GST, respectively, whilst BDEs were significantly related to CAT. Chlordanes are a type of insecticide which effects are blocking the hydrolysis of the neurotransmitter acetylcholine. At the same time, it may be related to the generation of free radicals after metabolic activation with enzyme induction of P450 which resulting products may enter reversible oxidation (Lushchak, 2011). It must be noted that seasonal studies have confirmed the difficulty to predict individual antioxidant responses since opposite changes in different areas can occur when the same environmental prooxidant factors have a different regional influence (Bocchetti and Regoli, 2006).

5.4 Physiological biomarkers

SFG data obtained in the present study were of the same order of magnitude as those published in previous SMP surveys (Albentosa *et al*., 2012a; Bellas *et al*., 2014), in which similar standardized laboratory conditions were used (temperature and food quantity and quality). Moreover, in the present survey we have included, for the first time, an exhaustive study of the mussel biology from different sites. This biological approach allows us to deepen in the previously identified effect of nutritive condition on the SFG biomarker, which acts as a confounding factor masking the mussels´ physiological responses to pollution, and limits their use in monitoring programs (Albentosa *et al*., 2012a; Bellas *et al*., 2014).

The physiological rate with the greatest effect on the SFG estimation is AR, which integrates two different physiological processes: ingestion and food absorption. Ingestion is directly obtained from CR and is the component of the energy budget most affected by pollution (Beiras *et al*., 2012; Howell *et al*., 1984; Toro *et al*., 2003). Clearance rates are usually standardized to mussel size, not only for comparative purposes with literature, but also for the inevitable slight differences in individual size between sampling sites. In fact, CR should be standardized to gill size, as filtration is a function of gill area. However, the difficulties in measuring the gill area have led to the use of mussel size instead of gill area for CR standardization. Thus, the most general standardization of CR is to 1 g of mussel dry tissue weight using the corresponding allometric coefficient that relates CR to body weight (recently reviewed by Cranford *et al*., 2011). However, changes in body caused by seasonal changes in reserves or reproductive tissues are not related to gill size and this introduces standardization errors (Cranford *et al*., 2011). These potential sources of errors related to differences in condition indices were proved by Filgueira *et al*. (2008). In the present study we clearly evidenced important differences between mussel populations in both, reserves and gonadal tissues which are summarized in the several biological indices used. These spatial differences in body indices have been associated to differences in food availability and can lead to a misunderstanding of the CR data. Therefore, shell length standardization as proposed by several authors (Filgueira *et al*., 2008; Iglesias *et al*., 1996; Labarta *et al*., 1997), has been considered a more accurate surrogate for gill size.

In the present study, even when $CR_{L\text{-}st}$ were considered, a significant inverse relationship was still observed between feeding and mussel condition, whereas in previous studies on the same populations, we observed that the standardization to length removes the effect of mussel condition (Albentosa *et al*, 2012a) or, when the mussel condition range was too narrow, its overall influence on CR was less evident (Bellas *et al*., 2014). In this survey, an alternative approach was carried out and mussels from different trophic areas (Atlantic and Cantabrian coasts) were studied separately. In this sense, when food availability is high (Atlantic area) CR is not affected by mussel condition. On the contrary, when food is less abundant (Cantabrian area) a clear effect of mussel condition is observed on CR.

Feeding behaviour in bivalves is clearly influenced by food availability. The classical model documented in a large number of publications shows that CR reaches maximum values at low food concentrations and that increases in food availability lead to a decrease of CR (Bayne and Widdows., 1978; Bayne, 1998; Brillant and MacDonald, 2003; Cranford and Hill, 1999; Cranford *et al*., 1998; Hawkins *et al*., 1998, 2001; Navarro *et al*., 2003; Urrutia *et al*., 2001; Velasco and Navarro,2003; Ward *et al*., 2003; Winter, 1978). Our hypothesis to explain the influence of condition on CR in the studied area is that mussels with poor condition come from habitats with lower food availability and that, in these environments, mussels respond to food scarcity with higher CR in order to maximize food intake. When these mussels are transferred to constant laboratory food conditions they still maintain the high CR associated to the habitat of origin. Thus, it seems that mussels have an "ecological memory" from the place where they have been collected (Babarro *et al*, 2000; Bayne, 1993; Mallet *et al*., 1987) and that depuration time established by ICES protocol (24 h, Widdows and Staff, 2006) does not seem to be enough to eliminate this "ecological memory".

Regarding chemical pollution effects on CR, it appears that in the Atlantic area, CR is negatively influenced by the presence of organochlorines as HCHs, coinciding with previous studies (Bellas *et al*., 2014; Albentosa *et al*., 2012a). Although this pesticide has been related to the disruption of gills functioning at highly laboratory concentrations (Donkin *et al*. 1997), this relationship was not observed in Cantabrian mussels, which the highest HCHs concentration (sites 28, 33 or 37). Similarly, the positive relationship detected between CR and As content in Cantabrian mussels was not observed in the Atlantic coast, where mussels exhibited the highest As values (sites 3, 5 and 12). In fact, this correlation was opposite as expected, since pollutants are generally reported to inhibit feeding behavior (reviewed in Widdows & Donkin, 1991). Anyhow, recent studies conclude that As concentrations are relatively uniform over the same study area, and that As mussel levels do not really represent pollution of the surrounding waters (Besada *et al*., 2014). This positive effect of As on CR may be due to other variables which covariates with this element. In this sense, an increase in As mussel content during winter and spring that might be related to the low nutritional state of mussels in these seasons has been previously described (Klarić *et al* 2004). This

hypothesis is in agreement with the higher CR observed in poor-condition mussels detected in the present study.

With regard to food absorption, mussels in the present survey showed a wide range of AE in comparison with previous studies (Albentosa *et al*., 2012a; Bellas *et al*., 2014). It is worth highlighting the very low AE values observed in sites from the Atlantic coast (e.g. sites 8, 14, 21 or 22), which clearly determined lower SFG values. However, low AE values were observed in low polluted sites, and no relationships between AE and biological parameters were identified. Therefore an alternative hypothesis needs to be considered. Low AE was obtained when faecal organic content was high and, if we consider that all mussels were studied under the same food quality conditions (i.e. the same food organic content), differences in AE should be related to digestive gland functioning. Nevertheless, results obtained in our laboratory (unpublished data) suggest that previous food conditions may influence the AE measurement carried out following the ICES protocol (Widdows and Staff, 2006). Faeces produced by mussels between time of arrival at the lab and the following day, when physiological determinations are carried out, were discarded in order to avoid the above mentioned effect. However, it might be possible that this purging time could not be enough for mussels from habitats with high and organically-enriched food availability. Actually, further experimental studies are being carried out in our lab in order to clarify this methodological issue.

Metabolic costs were mainly related to biological parameters instead of pollution, and again, these relationships have been highlighted only in Cantabrian areas. In this areas, significantly higher RR were recorded in mussels with lower ST. Some authors (Frew *et al*., 1989; Yap *et al*., 2003) consider that ST is an indicative of mussel age, so it might be stated that mussels' metabolism was higher in younger individuals. In addition, respiration is a physiological process proportional to a power of the body size, which in some way reflects the age of the organism. Allometric coefficients relating RR with body size are lower than 1, signifying that metabolic rate falls with increasing body size (Bayne and Newell, 1983) and, if we accept that for the same mussel population, smaller animals are younger than larger specimens, oxygen consumption rates would be higher in younger animals. What is not so clear is whether the same pattern occurs when organisms of the same body size have different ages, as the ones used in the present study. Studies on mussels from the same population with different age (Sukhotin and Pörtner, 2001; Sukhotin *et al*., 2003) documented an important decrease in oxygen consumption with age, which reflects the drop in mitochondrial respiration. Alterations of other physiological processes as filtration rate (Sukhotin *et al*, 2003), reproductive success (Sukhotin and Flyachinskaya, 2009) or growth rates (Abele *et al*, 2009) due to aging have also been described.

As a result of the integration of the above-mentioned physiological processes into the energy balance equation, SFG was clearly related to mussel condition. Higher SFG values were obtained in mussels with a poorer condition, reflecting the effect of mussel condition on absorption rates described before. Relationships observed in the

Atlantic mussels between some pollutants, as Pb or As and SFG are of different nature. Since Pb levels in Atlantic mussels are low and significantly lower than in the Cantabrian ones, an effect of this pollutant on SFG should be discarded. With regard to As another variable, which correlates with As levels, might be responsible for the detected relationship.

5.5. Comparative effects of biology and pollution on biomarker responses

In order to obtain an overview of the relative influence of biological and pollution variables on both sets of biomarkers, physiological and biochemical, a Mantel´s test was performed. For that purpose, the physiological (CR, AE, RR) and the biochemical (CAT, GST, GR, LPO) parameters were integrated in distance matrices. Then, each biomarker matrix was related with the biological matrix and with the pollutant matrix, being the results of these analyses shown in Table 11. No significant overall relationship was observed between biomarkers and biology or pollution ($p>0.05$), in the Atlantic area. On the contrary, a significant overall effect of biology on both biochemical ($r=0.420$, $p<0.05$) and physiological ($r=0.600$, $p<0.01$) biomarkers was evidenced in the Cantabrian coast. However, the test did not detect any relationship between the pollution and the different biomarker matrices.

Table 11. Mantel´s test analysis for biochemical and physiological biomarkers with biological and chemical variables for the two coasts studied.

Coast		Biochemical biomarkers vs		Physiological biomarkers vs	
		Biology	Pollution	Biology	Pollution
Atlantic coast	r	0.003	-0.090	-0.027	-0.169
	p-value	*0.980*	*0.540*	*0.840*	*0.182*
Cantabrian coast	r	0.420	-0.155	0.600	-0.077
	p-value	***0.018***	*0.490*	***0.006***	*0.720*

Many non-pollution related variables, as the ones considered here (nutritional state, metabolic activity, reproductive development or age), seem to act as confounding factors, modifying the expected and widely described biomarker responses to pollution (Van der Oost, 2003). When a wide area is monitored as in the SMP, strong differences occur in habitats where mussels are sampled. That determines mussel physiology and consequently those biological parameters used as pollution biomarkers. Among these habitat differences, food availability is one of the most important factors affecting general animal state for its influence on gonadal development (Bayne, 1976; Barber and Blake, 2006), energy balance (Bayne 1973; Albentosa *et al*. 1012b), and nutrient composition (Navarro *et al*., 1989; Orban *et al*., 2002; Albentosa *et al*, 2007). Furthermore, the effect of these confounding factors becomes crucial when comparing sites from a highly productive area, as the Atlantic Galician Rías, with sites from less productive areas, as the Cantabrian coast.

6. Conclusion

In previous studies we have reported that some biological variables hinder the establishment of relationships between pollutant concentrations present in the environment and their biological effects on organisms. Therefore, we recommended the incorporation of the analysis of a broad range of biological variables in pollution studies, over a wide range of environmental conditions. The present study follows this approach, in order to comprehend and characterize in detail how the variability of biomarker responses in mussels from two different oceanographic regions is explained by biological variables and by pollutant bioaccumulation.

As stated previously, a clear difference in pollution levels was observed between the two regions, with the Cantabrian area showing the highest average pollutant concentrations. In addition, it was shown that the accumulation of certain organic pollutants such as PCBs, DDTs and BDEs, were higher in younger individuals, which could be related to its higher activity in comparison with older individuals. Although results presented here indicate that the bioaccumulation of different pollutants (metals-Cu, Zn, Cd, As-, chlordanes, HCHs) significantly affects the molecular and physiological biomarkers measured, these relationships were masked by 'confounding factors'.

As expected, a great geographical variability was observed for the studied biological variables, including the nutritional state of mussels along with their biochemical composition, gonadal development and age. This variability was mainly linked to differences in food availability between both regions. In general, antioxidant biomarkers in mussels presented higher values in mussels with poorer condition and with lower sexual maturity index, which evidences an inverse relationship between antioxidant enzymes and the nutritional state of the organism. LPO was positively related to the nutritional status and, therefore, with higher metabolic costs, to their associated ROS generation. Mussel condition was also inversely related to CR, and therefore to SFG, suggesting that mussels keep an "ecological memory" from the habitat where they have been collected. On the other hand, RR was positively related to ST, which indicates that younger mussels present higher metabolic rates than older individuals.

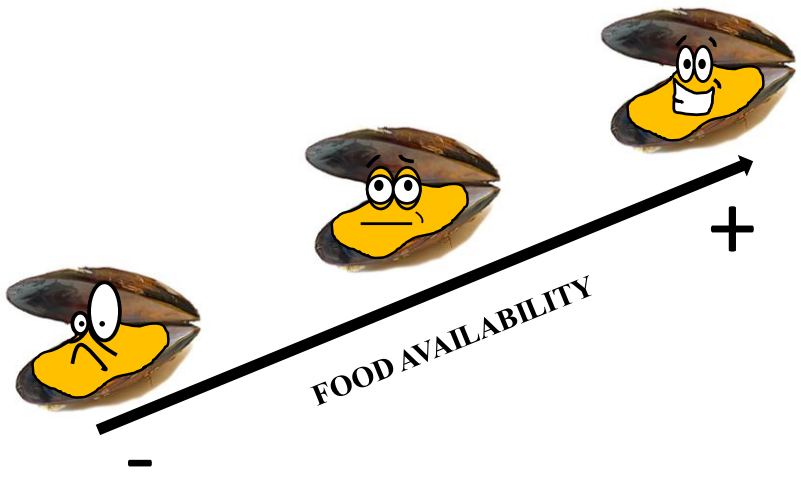
Finally, no overall relationship was observed between pollution and biomarkers, but a significant overall effect of biological variables on both biochemical and physiological biomarkers was evidenced in the Cantabrian coast; this relationship was not observed in the Atlantic area. Therefore, when a wide range of certain environmental factors, as food availability, coexist in the same monitoring program, it determines a great variability in mussel populations which mask the effect of contaminants on biomarkers.

The measure of a complete set of biological parameters as in the present study provides useful information to determine the physiological state of the mussels and its influence on the biological processes used as pollutant biomarkers. As recommended by

Nahrgang *et al*. (2013), it should be highly desirable to include, in addition to mussel biological parameters, the measurement of physico-chemical variables of the environment for a correct interpretation of biomarker responses. Both, biological and environmental parameters should be considered complementary to biomonitoring procedures. On another hand, laboratory experiments where biological and pollution variables can be easily isolated, should be carried out with the purpose to determine biomarker responses to contaminants over a wide range of environmental and intrinsic conditions.

CHAPTER 2

Condition I: Nutritive state-Quantity



Carmen González Fernández, 2016

The research presented in this chapter forms part of the following publication:

González-Fernández, C., Albentosa, M., Campillo, J. A, Viñas, L., Romero, D., Franco, A., Bellas, J., 2015. Effect of nutritive status on Mytilus galloprovincialis pollution biomarkers: Implications for large-scale monitoring programs. Aquat. Toxicol. 167, 90–105.

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ABSTRACT

Biomarkers have been extensively used in monitoring programs aiming to assess the biological effects of pollutants on marine organisms and determining the environmental state. Data obtained from these programs are sometimes difficult to interpret for the large amount of natural variables affecting biological processes, which could act as confounding factors on biomarker responses. The main aim of this work was to identify the effect of one of these variables, food availability, and consequently, the mussel nutritive state, on biomarker responses. For that purpose, mussels (*Mytilus galloprovincialis*) were conditioned to three different food rations for 2 months in order to create three mussel nutritive states and, later on, each state was exposed to three nominal concentrations of fluoranthene (FLU) for three weeks. A battery of biomarkers was considered in this study to cover a wide range of organism responses: physiological (scope for growth –SFG-) and biochemical (superoxide-dismutase –SOD-, catalase –CAT-, glutathione reductase –GR-, glutathione peroxidase –GPx-, glutathione-S-tranferase –GST- and lipid membranes peroxidation –LPO-). The results obtained evidenced that most of the studied biomarkers (SFG, SOD, CAT and GPx) were strongly affected by the mussel nutritive state, showing higher values at lower states, whereas the effect of toxic was not always evident, masked by a nutritive state effect. This paper demonstrates that toxicants are not the only source of variability modulating pollution biomarkers, and confirms nutritive state as a major factor altering biochemical and physiological biomarkers.

Keywords: Biomonitoring, mussels, confounding factors, nutritive state, biomarkers, fluoranthene

1. Hypothesis

Chapter 1 revealed that mussels' biomarker responses are dependent of mussel condition which acts as a confounding factor hindering the use of biomarkers in monitoring programs. Mussel condition is dependent on both, nutrition and reproduction, which are closely associated. Nutritive variability in natural environments is related to both, food quantity and quantity. The hypothesis of the present chapter is that mussel pollution biomarkers are affected by the amount of food available in the marine environment.

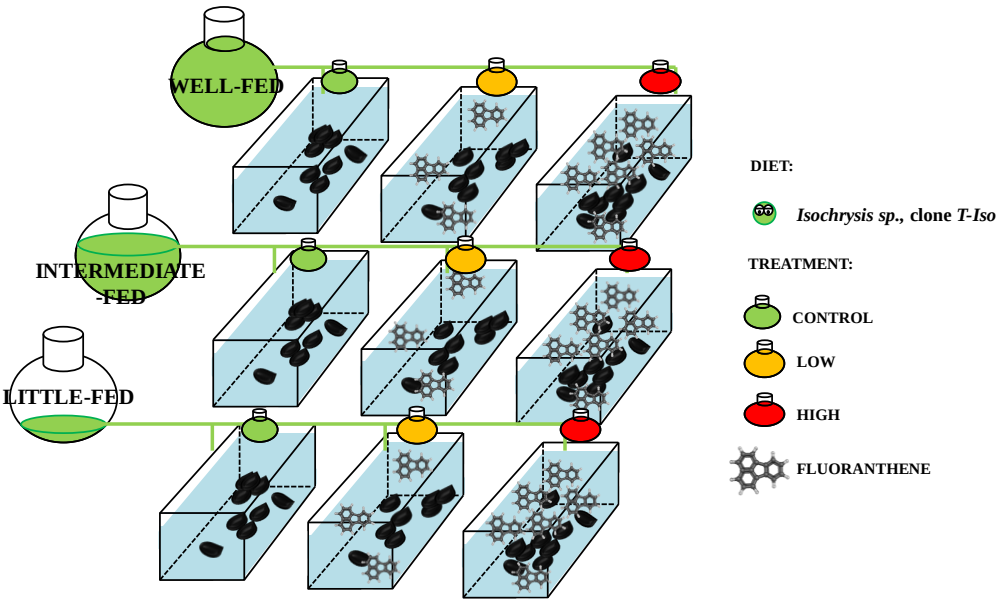
2. Objectives

The overall objective of this chapter was to assess, under laboratory conditions, the biomarker responses to pollution of mussels with different quantitative nutritive conditions. The experiment was carried out in early summer, a time of the year far from gametogenesis (autumn) or spawning (spring), in order to isolate the experimental variable nutritive state from gonadal development (reproductive state).

The specific objectives of this chapter were to identify i) the effect of mussel nutritive state on a battery of biochemical and physiological biomarkers, ii) the combined effect of pollutant and mussel nutritive state on biomarkers responses, and iii) the consequences of the obtained results in large-scale monitoring programs.

3. Summary of experimental design

Mussels were conditioned to three different food rations for 2 months in order to create three mussel nutritive states and afterwards, each state was exposed to two nominal concentrations of fluoranthene (FLU).



4. Results

4.1. Nutritive-conditioned mussels

After 56 days of conditioning, mussel soft tissue dry weights differed between nutritive conditions. NS-1 and NS-3 mussels displayed the lowest and the highest tissues dry weights, 182.0 and 344.6 mg respectively, being NS-3 almost the double of NS-1, whereas NS-2 mussels showed an intermediate weight (248.8 mg). Mean and standard deviation of mussel biological indices after the conditioning are shown in Table 12. Indices (CI_{vol}, CI_{shell}, MI, SMI, HI) were statistically different depending on nutritive state (ANOVA, $p<0.01$) with higher values for NS-3 mussels which decreased with the nutritive state. GI showed an opposite pattern, with NS-1 mussels showing higher values. It needs to be highlighted that, although NS-2/NS-3 mussels displayed higher SMI, this values are really low (close to 1) in a scale from 0 to 4.

ANOVA results of mussel biochemical components showed a strong significant effect ($p<0.01$) of mussel nutritive state in all components (Table 11). CH is the component more affected by condition increasing 8-fold from NS-1 to NS-3. The same pattern was observed in PROT content but to a lesser extent. LIP was also affected by mussel nutritive state with NS-1 mussel showing the lowest content, with less than half of the lipids observed in the other mussels.

Table 12. Biological parameters (means and standard deviations) of mussels (*M. galloprovincialis*) with 3 nutritional states (NS-1, NS-2, NS-3) and exposed to two nominal concentrations of fluoranthene (Low and High). CI_{Vol}: Volumetric condition index, CI_{Shell}: Shell condition Index, GI: Gill Index, GSI: Gonadosomatic Index, SMI: sexual maturity index, HI: Hepatosomatic Index, LIP: lipids, CH: carbohydrates and PROT: proteins. Biochemical composition of mussel after conditioning (mg/ind) and after exposure (mg/g organic matter). Significant differences between exposure treatments (lowercase letters) and mussel nutritive conditions (capital letters) were obtained from the multiple range test (LSD) after one-way ANOVA at a significant level of $p<0.05$.

Treatment	Mussel nutritive state	Biological Indices						Biochemical composition		
		CI_{Vol}	CI_{Shell}	GI	GSI	SMI	HI	LIP	CH	PROT
					After conditioning time					
Initial	NS-1	2.1±0.2 ^{a}	5.5±0.8 ^{a}	19.2±2.3 ^{c}	4.2±2.8 ^{a}	0.00±0.00 ^{a}	24.0±2.4 ^{a}	19.7±1.0 ^{a}	11.6±1.7 ^{a}	117.0±0.6 ^{a}
Initial	NS-2	2.6±0.3 ^{b}	7.2±0.8 ^{b}	16.6±1.7 ^{b}	12.1±3.0 ^{b}	0.57±0.94 ^{b}	27.5±3.5 ^{b}	40.9±4.9 ^{b}	44.9±0.5 ^{b}	128.1±4.7 ^{b}
Initial	NS-3	3.5±0.6 ^{c}	8.8±1.7 ^{c}	12.8±2.8 ^{a}	19.5±3.2 ^{c}	0.64±0.93 ^{b}	26.4±2.7 ^{b}	43.9±5.3 ^{b}	81.3±1.4 ^{c}	175.6±2.8 ^{c}
					After exposure time					
Control	NS-1	2.0±0.2^{aA}	5.2±0.6^{aA}	19.5±1.8^{aC}	7.8±3.4^{aA}	0.00±0.00^{aA}	23.6±3.2^{aA}	182.8±6.6^{aC}	129.8±18.5^{abA}	687.3±12.4^{aC}
Low	NS-1	2.1±0.2^{aA}	5.3±0.7^{aA}	21.4±2.4^{aC}	7.8±2.8^{aA}	0.00±0.00^{aA}	24.3±3.0^{aA}	181.3±9.0^{aB}	140.7±20.4^{bA}	677.9±25.0^{aC}
High	NS-1	1.8±0.2^{aA}	5.2±0.6^{aA}	20.5±2.2^{aC}	9.0±2.2^{aA}	0.00±0.00^{aA}	23.3±3.3^{aA}	165.8±13.6^{aB}	93.8±18.7^{aA}	740.3±29.7^{bC}
Control	NS-2	2.8±0.5^{aB}	7.5±1.1^{aB}	16.4±1.5^{aB}	13.6±3.6^{aB}	1.20±0.86^{aB}	27.1±3.2^{aB}	159.5±9.2^{aB}	290.6±18.9^{bB}	549.8±14.2^{aB}
Low	NS-2	2.7±0.3^{aB}	7.4±1.0^{aB}	16.2±1.8^{aB}	12.8±4.2^{aB}	1.08±0.86^{aB}	28.4±2.2^{aB}	157.4±15.8^{aA}	259.0±26.1^{abB}	583.6±27.4^{abB}
High	NS-2	2.8±0.4^{aB}	7.6±1.4^{aB}	15.7±1.8^{aB}	15.6±2.9^{aB}	1.00±0.91^{aB}	27.4±4.9^{aB}	152.0±10.1^{aA}	236.6±16.2^{aB}	611.4±12.5^{bB}
Control	NS-3	3.1±0.5^{aC}	8.0±1.5^{aB}	13.5±2.0^{aA}	20.0±4.6^{aC}	1.20±1.42^{aB}	23.5±4.0^{aA}	116.3±8.7^{aA}	408.3±3.4^{bC}	475.4±11.78^{aA}
Low	NS-3	3.5±0.9^{aC}	8.9±1.9^{aC}	13.2±3.5^{aA}	24.2±7.8^{aC}	0.79±0.70^{aB}	23.1±4.6^{aA}	105.4±12.0^{aA}	378.8±47.1^{bC}	515.7±43.5^{aA}
High	NS-3	2.9±0.7^{aB}	8.0±1.7^{aB}	13.8±2.9^{aA}	22.9±6.3^{aC}	1.14±0.95^{aB}	23.2±4.4^{aA}	124.5±19.9^{aA}	317.1±3.2^{aC}	558.4±18.9^{bA}

Figure 26 shows the differences in the initial values of the total organic matter and of biochemical components in mussel tissues after the conditioning period. Nutritive-stressed mussels (NS-1) showed lower values than at the beginning of the experiment of all components (OM, LIP, CH and PROT) which resulted in a negative growth during conditioning. Conversely, well-fed NS-2 and NS-3 mussels showed a positive growth and higher values of their biochemical components, with CH exhibiting the highest increase. In fact, NS-3 mussels showed almost double CH values than NS-2-mussels, and more than six times higher than NS-1 mussels. LIP showed approximately the same value for NS-3 and NS-2 mussels, and negative values for NS-1-mussels. Furthermore, PROT showed a positive value for NS-3 mussels, whereas NS-2 showed values close to zero and NS-1 mussels showed a negative value.

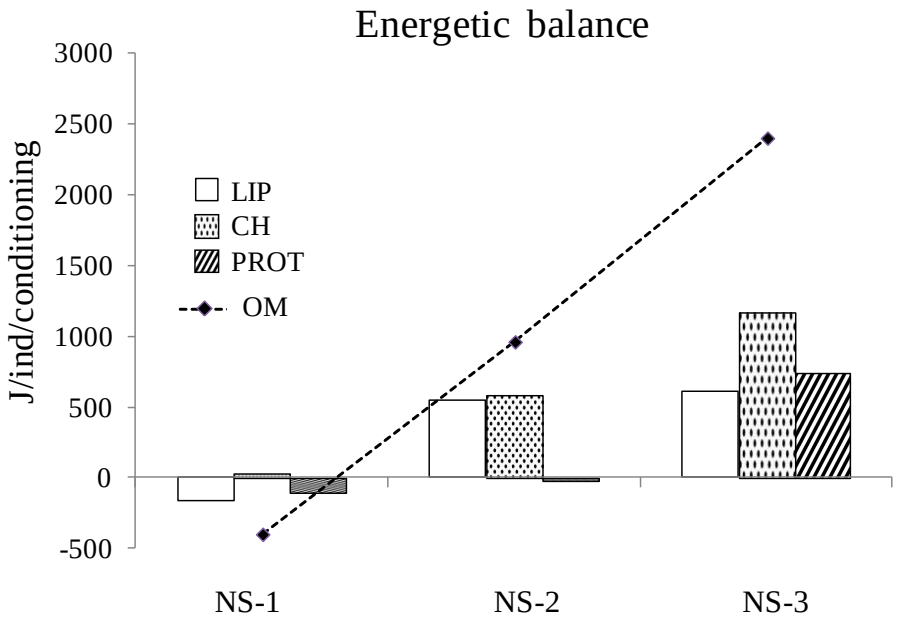


Figure 26. Energetic balance (estimated from the differences in body tissues between initial and final weights) of wild mussels (M. galloprovincialis) with different nutritional state: negative (NS-1), low growth (NS-2) and high growth (NS-3) after 56 days of conditioning to three different food rations. Data were expressed as increases in Joules per individual and per the whole period. Measurements were in total organic matter (OM), lipids (LIP), carbohydrates (CH) and proteins (PROT).

4.2. Exposure measurements

Mussel absorption efficiencies (AE) during the experimental period (conditioning and exposure) were calculated and shown in Figure 24. In this case, AE were indirectly estimated from total ingested organic matter by each condition mussels during this period, real mussel growth and respiration rates (detailed in section 2.2). Figure 27 evidenced different AEs with maximum values in NS-1 and decreased with higher mussel nutritive state. Two-factor ANOVA performed on AE data, showed a significant effect of nutritive state on mussels AE ($p<0.01$) whereas no significant effect

of the toxicant was detected.

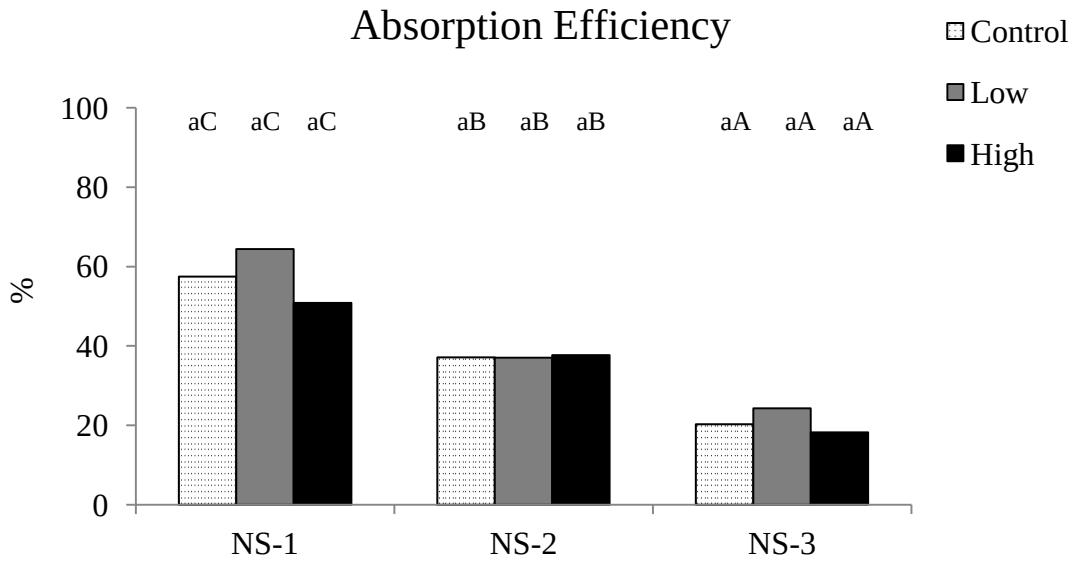


Figure 27. Mussel absorption efficiencies of wild mussels (*M. galloprovincialis*) with different nutritional state: negative (NS-1), low growth (NS-2) and high growth (NS-3) (*M. galloprovincialis*) during the experimental period (conditioning and exposure). Significant differences between exposure treatments (lowercase letters) and mussel nutritive conditions (capital letters) were obtained from the two-way ANOVA analysis at a significant level of $p<0.05$.

Ingestion test results showed no significant differences between the three weeks of exposure, so all ingestion data collected during this period were considered as replicates of each group (data not shown). Mussel ingestion rate (IR) showed a significant effect of mussel nutritive state, that was specially evidenced in NS-3 mussels which displayed the lower IR (NS-3<NS-2=NS-1). On the contrary, not important effect of FLU on IR was observed (two-way ANOVA, $p<0.01$), and only NS-1 mussels showed a significant increase of IR at High FLU concentration.

4.3. End-point measurements

4.3.1 Biological measurements

Means and standard deviations of mussel biological indices and biochemical components after exposure are shown in Table 12. Two-way ANOVA showed that mussel biological indices were statistically different depending on mussel nutritive state in a similar way as in the conditioning period (described in 3.1.). A significant effect of nutritive state was observed in all biological indices whereas the FLU exposure factor was not significant ($p>0.05$). F values from the two-way ANOVA were considerably higher for the nutritive state factor (F_{NS}) than for toxicant exposure (F_T): CI_{vol} (F_{NS}/F_T=67.5/2.6), CI_{shell} (F_{NS}/F_T=72.0/0.9), GI (F_{NS}/F_T=106.7/0.5), GSI (F_{NS}/F_T=110.3/2.3), SMI (F_{NS}/F_T=26.4/0.6), and HI (F_{NS}/F_T=18.5/0.4).

Two-way ANOVA also evidenced that both factors, mussel nutritive state and toxicant exposure, have an effect on mussel biochemical components. In a similar way as in the conditioning period, biochemical components were statistically different between nutritive state with higher content ($p<0.01$) of CH as food ratio increases, and with a 5-fold difference between well-fed (NS-3) and nutritive-stressed mussels (NS-1). PROT and LIP also showed higher values as nutritive state increases, although they were of a lower order of magnitude than CH. The effect of mussel condition was higher than that of FLU exposure also for the biochemical components as it can be concluded from the F test of the multifactor ANOVA: LIP (F_{NS}/F_T=57.7/0.7), CH (F_{NS}/F_T=521.3/36.1) and PROT (F_{NS}/F_T=222.3/31.4). Despite this, there was a significant effect ($p<0.01$) of toxic on the CH values which decreases at high FLU concentrations (Table 12).

4.3.2 Biomarkers

4.3.2.1 Bioaccumulation

Mussel bioaccumulation data are shown in Table 13. Two-factor ANOVA showed a dose-dependent significant increase of FLU accumulation in mussel tissues ($p< 0.001$). Moreover, the accumulation of FLU was different according to their nutritive state; the lower mussel conditions (NS-1 and NS-2) displayed the highest FLU tissue accumulation at High exposure dose, whereas at Low exposure dose, NS-2 mussels showed the highest FLU accumulation.

Table 13. Fluoranthene (FLU) accumulation in mussel tissues (means and standard deviations) of mussels (*M. galloprovincialis*) with 3 nutritive states (NS-1, NS-2, NS-3) and exposed to two nominal concentrations of FLU (Low and High). Significant differences between exposure treatments (lowercase letters) and mussel nutritive conditions (capital letters) were obtained from the multiple range test (LSD) after one-way ANOVA at a significant level of $p<0.05$.

Treatment	Mussel nutritive state	Bioaccumulation	
		µg FLU g^{-1} dw $^{-1}$	Efficiency (%)
Control	NS-1	0.0 ± 0.0^{aA}	0.0 ± 0.0^{aA}
Low	NS-1	9.5 ± 2.2^{bA}	7.7 ± 1.8^{bA}
High	NS-1	198.3 ± 4.6^{cB}	8.8 ± 0.2^{bB}
Control	NS-2	0.0 ± 0.0^{aA}	0.0 ± 0.0^{aA}
Low	NS-2	15.3 ± 0.5^{bB}	12.4 ± 0.4^{bB}
High	NS-2	221.7 ± 31.3^{cB}	11.1 ± 1.6^{bC}
Control	NS-3	0.0 ± 0.0^{aA}	0.0 ± 0.0^{aA}
Low	NS-3	7.3 ± 2.1^{bA}	5.7 ± 1.6^{bA}
High	NS-3	162.5 ± 6.61^{cA}	6.7 ± 0.3^{bA}

On another hand, accumulation efficiency of FLU did not show any significant difference between exposure treatments (Low and High), whilst significant differences were found between conditions, highlighting that NS-2 mussels accumulated around 12% of the offered toxic (12.4% for Low and 11% for High), that was almost double than the other two mussel conditions. NS-1 mussels showed an efficiency of 7.7% for Low and 8.8% accumulation for High and the lowest percentages of efficiency were observed in NS-3 mussels, with 5.7 and 6.7% for Low and High respectively.

4.3.2.2 Physiological biomarkers

Mean values of physiological rates used for SFG estimation and their standard deviations are shown in Table 14 for the 9 experimental groups. In general, NS-1 mussels showed the highest SFG values, being statistically different from NS-2 and NS-3 mussels. In fact, SFG of NS-1 mussels were 3 and 5 times higher than NS-2 and NS-3 mussels, respectively. Regarding toxic effect, only NS-1 mussels showed an effect of FLU, being mussels at Low which evidenced highest SFG.

Table 14. Physiological parameters (mean and standard deviations) of mussels (*M. galloprovincialis*) with 3 nutritional states (NS-1, NS-2, NS-3) and exposed to two nominal concentrations of fluoranthene (Low and High). Physiological rates were measured under standardized laboratory conditions (15ºC, filtered seawater 1µm, 0.8 mg/l of algal cells and 0.5 mg of inorganic matter/l). $CR_{L\text{-}st}$: clearance rate standardized to a mussel of 60 mm length, $CR_{Gill\text{-}st}$: clearance rate standardized to a mussel of 1g of gill dry weight, $CR_{W\text{-}st}$: clearance rate standardized to a mussel of 1g of total dry weight, AE: absorption efficiency, RR: respiration rate and, SFG: scope for growth. CRs were expressed as liters per each specific standard individual and per hour and RRs and SFGs data were expressed as joules per standard individual of 1 g of dry weight per hour. Significant differences between exposure treatments (lowercase letters) and mussel nutritive conditions (capital letters) were obtained from the multiple range test (LSD) after one-way ANOVA at a significant level of $p<0.05$.

Treatment	Mussel nutritive state	$CR_{L\text{-}st}$	$CR_{Gill\text{-}st}$	$CR_{W\text{-}st}$	AE	RR	SFG
		L/h			%	J/g/h	
Control	NS-1	2.6 ± 0.3^{aC}	16.1 ± 2.9^{aC}	5.3 ± 0.8^{aC}	62.4 ± 8.4^{aA}	14.4 ± 1.2^{bC}	43.6 ± 12.3^{aC}
Low	NS-1	3.5 ± 0.7^{aB}	18.6 ± 4.1^{aB}	6.4 ± 1.3^{aB}	71.8 ± 2.7^{bA}	12.1 ± 0.9^{aB}	69.0 ± 14.8^{bB}
High	NS-1	3.0 ± 1.2^{aB}	17.9 ± 7.0^{aB}	6.2 ± 2.5^{aB}	66.6 ± 4.7^{abB}	14.1 ± 0.9^{bB}	58.4 ± 28.6^{abB}
Control	NS-2	1.9 ± 0.8^{abB}	10.0 ± 3.5^{abB}	3.0 ± 1.1^{abB}	67.0 ± 8.7^{aA}	10.2 ± 2.3^{aB}	25.2 ± 14.3^{aB}
Low	NS-2	1.3 ± 1.3^{aA}	7.3 ± 7.8^{aA}	2.1 ± 2.2^{aB}	81.4 ± 7.5^{bB}	10.4 ± 3.0^{aB}	18.7 ± 26.2^{aA}
High	NS-2	2.7 ± 1.4^{bB}	15.0 ± 7.8^{bB}	4.3 ± 2.2^{bB}	64.5 ± 10.0^{aB}	13.2 ± 3.8^{bA}	37.5 ± 24.2^{aB}
Control	NS-3	0.8 ± 0.2^{aA}	4.5 ± 1.0^{aA}	1.1 ± 0.2^{aA}	76.8 ± 10.7^{bB}	7.0 ± 1.5^{aA}	8.3 ± 4.8^{aA}
Low	NS-3	1.1 ± 0.7^{aA}	5.4 ± 3.2^{aA}	1.3 ± 0.9^{aA}	74.6 ± 8.6^{bAB}	8.3 ± 1.6^{bA}	8.4 ± 9.3^{aA}
High	NS-3	1.2 ± 0.9^{aA}	6.9 ± 5.7^{aA}	1.8 ± 1.6^{aA}	36.4 ± 20.9^{aA}	11.0 ± 1.3^{cA}	3.0 ± 16.4^{aA}

Two-factor ANOVA analyses on physiological biomarkers showed that both factors (mussel nutritive state and FLU exposure) influenced mussel physiology. However, the effect of mussel nutritive condition was notably higher than that of FLU exposure in most of the physiological biomarkers considered, as it is shown in Figure 28 where the F-test values were plotted for each factor and for the interaction of both of them.

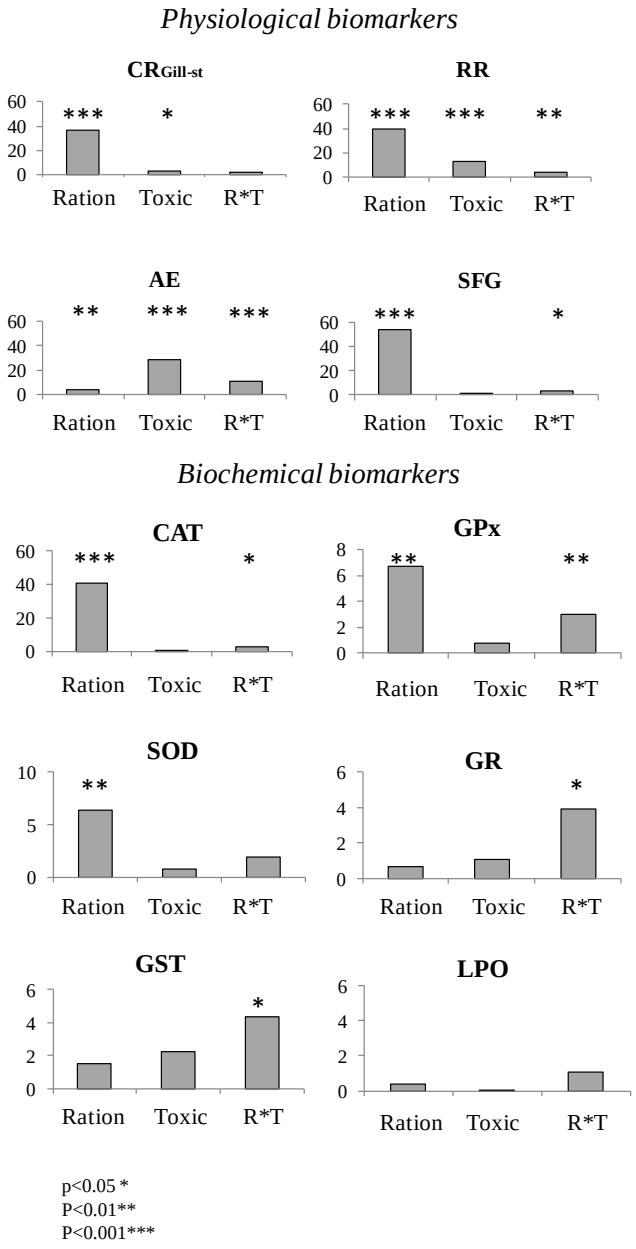


Figure 28. Results for the two-factor ANOVA on the physiological and antioxidant biomarkers responses. Bars showed the value of the F-test from the multifactor ANOVA being one factor the mussel nutritional state (Ration) obtained supplying different food rations and the other factor the exposure to FLU at two doses (Toxic). Interaction F values between both factors (R*T) were also plotted. Significance of each F value was noted as *, ** or *** for a significance level of $p<0.05$, $p<0.01$ or $p<0.001$, respectively.

Regarding control mussels, clearance rates in their different standardizations ($CR_{L\text{-}st}$, $CR_{Gill\text{-}st}$, $CR_{W\text{-}st}$) were strongly affected by mussel nutritive state (Two-way ANOVA, $p<0.001$). Mussels with the highest nutritive state (NS-3) exhibited the lowest CR which inversely increased with low nutritive state. FLU had also a significant effect on CRs (see Figure 28) but this effect was only noticed in NS-2 mussels. In these mussels and contrary to the expected results, clearance rates increased at High exposure whereas Low exposure caused a decrease of CR, being these values even lower than those in control mussels, although this difference was not significant. All the CR standardizations ($CR_{L\text{-}st}$, $CR_{Gill\text{-}st}$, $CR_{W\text{-}st}$) evidenced the same pattern.

A two-factor ANOVA analysis showed that both factors influenced the absorption efficiency (AE) of mussels, as well as the interaction. However, a stronger effect was caused by the toxicant (Figure 28). Considering control mussel condition, significantly AE higher values were found in NS-3 mussels, despite that differences were not too large (62% as opposed to 76%). In addition, a significant effect of the toxicant was noticed in all conditions but in a different way (significant interaction). At NS-1 and NS-2 conditions and disagreeing with expected results, AE was significantly higher at Low FLU concentrations whereas no differences were detected between Control and High FLU. However, similarly to mussel condition effect on AE, these higher AE were not too large to have an effect on mussel balance. On the contrary, at NS-3 condition there was a significantly high decrease of AE at High exposure: 36% at High whereas AE registered values of 75% at Low or Control mussels.

On another hand, two-factor ANOVA analysis showed that RRs were affected by both factors and by their interaction (Figure 28). With regard to mussel nutritive state, RRs in control mussels evidenced the same pattern than CRs, showing NS-1 mussels the highest values. Respiration rates also increased with FLU exposure, being significantly higher at High exposure, except for NS-1 mussels. This effect was more evident in NS-3 mussels (Control<Low<High).

4.3.2.3 Biochemical biomarkers

Biochemical biomarkers means and standard deviations are shown in Table 15. Two-factor ANOVA analyses showed that only mussel nutritive state influenced antioxidant biomarkers (Figure 28), although this influence was different depending on the toxicant exposure. Statistical analyses showed that SOD activity was only affected by nutritive state ($p<0.01$) decreasing at highest mussel condition. The same statistical analysis showed that both factors, NS and NS*T, affected CAT activity (Figure 28). CAT values significantly decrease with higher mussel nutritive state. Furthermore, this activity was affected by the toxicant but only in NS-2 mussels, which displayed the highest value of CAT at Low FLU exposure. GPx evidenced a similar pattern than CAT, being affected not only by nutritive state ($p<0.01$) but also by NS*T. Regarding mussel condition, control mussels showed lower GPx activity at high mussel condition. The effect of FLU was only evident in NS-3 mussels; Low exposed mussels displaying higher GPx activity values. In contrast to the antioxidant activities mentioned before, GR was not affected by nutritive state and the only significant parameter of the analysis

was the interaction of NS*T ($p<0.05$). This interaction revealed an effect of FLU exposure, but only in NS-3 mussels with higher GR values at Low exposure.

GST activity also showed the effect of both factors. GST activity for control mussels was lower at high (cuidado con highest, higher or high, no se que quieres decir. Ojo que se repite) mussel nutritive state, which was in agreement with results of the antioxidant activities. Moreover, the toxic effect was evident in all mussel conditions but with a different trend. NS-1 mussels showed a lower GST activity at Low FLU exposure, whereas in high mussel conditions, a higher GST activity was observed at Low exposure.

LPO activity did not show any significant ($p>0.05$) effect of nutritive state or toxicant exposure.

Table 15. Summary of biochemical biomarkers (means and standard deviations) of mussels (*M. galloprovincialis*) with 3 nutritional states (NS-1, NS-2, NS-3) and exposed to two nominal concentrations of fluoranthene (Low and High). SOD: Superoxide-dismutase, CAT: Catalase, GST: Glutathione-S-transferase, GR: Glutathione Reductase, GPx: Glutathione peroxidase and LPO: Lipid Peroxidation. Significant differences between exposure treatments (lowercase letters) and mussel nutritive conditions (capital letters) were obtained from the multiple range test (LSD) after one-way ANOVA at a significant level of $p<0.05$.

Treatment	Mussel nutritive state	SOD	CAT	GR	GPx	GST	LPO
		U min^{-1} mg $prot^{-1}$	µmol min^{-1} mg $prot^{-1}$	nmol min^{-1} mg $prot^{-1}$			nmol MDA mg $prot^{-1}$
Control	NS-1	84.49 ± 21.1^{aB}	88.9 ± 19.6^{aC}	37.1 ± 11.7^{aA}	4.5 ± 1.6^{aB}	91.0 ± 3.1^{bB}	3.5 ± 1.6^{aA}
Low	NS-1	74.39 ± 9.5^{aA}	74.4 ± 5.7^{aB}	27.3 ± 6.2^{aA}	4.1 ± 1.6^{aA}	69.6 ± 9.9^{aA}	3.3 ± 1.2^{aA}
High	NS-1	93.30 ± 25.1^{aB}	82.5 ± 13.6^{aB}	42.5 ± 13.7^{aA}	4.1 ± 0.9^{aB}	91.2 ± 17.5^{bB}	3.8 ± 1.2^{aA}
Control	NS-2	64.90 ± 18.4^{aAB}	64.1 ± 18.2^{abB}	32.5 ± 6.5^{aA}	4.3 ± 2.0^{aAB}	51.6 ± 12.0^{aA}	3.3 ± 1.4^{aA}
Low	NS-2	84.71 ± 30.9^{aA}	75.9 ± 20.8^{bB}	43.0 ± 13.8^{aA}	2.5 ± 1.4^{aA}	86.4 ± 24.5^{bA}	4.0 ± 1.2^{aA}
High	NS-2	60.64 ± 11.5^{aA}	46.9 ± 13.6^{aA}	34.6 ± 8.6^{aA}	3.6 ± 1.2^{aB}	75.1 ± 12.9^{abAB}	2.9 ± 0.4^{aA}
Control	NS-3	51.97 ± 7.2^{aA}	25.3 ± 3.4^{aA}	31.9 ± 6.1^{aA}	2.3 ± 0.3^{aA}	70.5 ± 16.7^{abA}	3.3 ± 0.7^{aA}
Low	NS-3	68.32 ± 18.3^{aA}	30.7 ± 6.1^{aA}	49.4 ± 8.5^{bA}	3.8 ± 1.0^{bA}	102.5 ± 36.4^{bA}	2.9 ± 0.6^{aA}
High	NS-3	59.19 ± 7.6^{aA}	36.4 ± 21.3^{aA}	39.2 ± 13.4^{abA}	1.5 ± 0.7^{aA}	67.2 ± 10.1^{aA}	3.7 ± 0.8^{aA}

4.4. Integrative assessment

A PCA was carried out In order to integrate biomarker responses of mussels. The data set used in the analysis included all biomarkers studied: the biochemical biomarkers (SOD, CAT, GPx, GR, GST and LPO) and the three components that integrate the SFG assay ($CR_{Gill\text{-}st}$, AE and RR). The integration of all these variables in the PCA was represented by four principal components that explained 94.3% of the variance in the original data set. The most important principal component, PC1, explained 38.7 % of the total variance positively relating SOD, CAT, GPx, $CR_{Gill\text{-}st}$ and RR. High loadings (>0.7) were obtained in all cases, indicating a strong association between them. PC2 accounted for 22.2% of the total variance, and grouped GST and GR positively in a strong way (>0.8). PC3, explained 19.5 % of the total variance and only represented LPO; and finally, PC4 accounted for 14.8 % of the total variance and presented AE isolated. Figure 26 shows the graphic representation of PCA variables, evidencing a clear separation of the 3 mussel nutritive states. NS-1 mussels were located on the right side of axis 1, with higher values of SOD, CAT, GPx, $CR_{Gill\text{-}st}$ and RR, whereas NS-3 mussels were located on the left side of axis 1 showing lower values of these variables. NS-2, the intermediate mussel condition, was located between these two conditions. Regarding the treatment, Low of NS-2 and NS-3 conditions were located on the upper side of axis 2 showing higher values of GST and GR, as well as High and Control treatments of NS-1. Control and High treatments of NS-2 and NS-3 were located on the bottom side of axis 2 as well as Low treatment of NS-1 mussels showing lower GST and GR values.

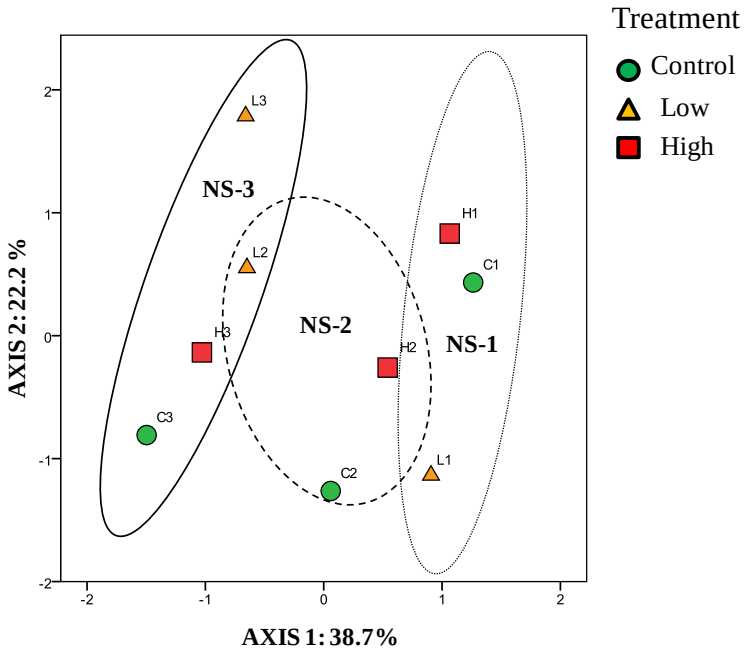


Figure 29. PCA analyses carried out with all biomarkers measurements: the biochemical biomarkers (SOD, CAT, GPx, GR, GST and LPO) and the three components that integrate the SFG assay ($CR_{Gill\text{-}st}$, AE and RR). Two components (Axis 1 and Axis 2) explaining 60.9 % of the total variance, were plotted. PCA analysis arranged mussels into three different groups according with their nutritive condition.

5. Discussion

5.1 Nutritive-conditioned mussels

After the long conditioning process (56 days), a range of mussel nutritive state was observed. Mussels fed with the lower food ration (NS-1) showed a clear decrease of mussel tissues compared to initial mussels. This decrease was observed in all of their biochemical components (Figure 23). In this condition, mussels catabolize their own tissues as an adaptive response to low food availability with the goal of obtaining energy and surviving. As it was described by Moore *et* at. (2004), dietary restriction induces autophagy which is the degradation of cellular components in lysosomes. Autophagy is considered not only a primary survival strategy against stress but is also intimately involved in cell physiology. This process has also been described under other environmental stressors such as hypoxia, hyperthermia or pollutants (Bayne *et al*., 1978; Moore and Halton, 1973; Moore *et al*., 1979, 1985, 2007). Under these conditions, the autophagic system is repeatedly triggered, removing altered cellular components, which contribute to increase lifespan through the maintenance of more efficient "cellular housekeeping" (Bergamini *et al*., 2003, Moore *et al*., 2007).

On the other hand, two anabolic mussel conditions were obtained with the increase of food rations, where mussels showed a positive growth: NS-2 mussels showed an increase in energetic reserves (lipids and carbohydrates) and maintained their structural tissues (proteins) and NS-3 mussels increased both energetic storages and structural components. In both cases, the highest increase was observed for carbohydrates as they are, particularly glycogen, the main source of energy in mussels (Bayne, 1973). Although mussels differed in biochemical component contents after the conditioning period, they did not show any significant difference in their reproductive state, which allowed us to isolate the effect of nutritive state without the common interference of reproductive state. Thus, a gradient of MI was observed with condition but all of them showed the same reproductive state with values from 0-1 in the SMI, which is considered as no ripe state. Mussels were collected at the end of spring which is the main spawning season in the Galician coast (Caceres-Martinez and Figueras, 2007). The exposure experiment was carried out in summer, after being conditioned during late spring. Thus, mussels arrived with immature gonads and remained in the same state even under the highest energetic food ratio. Energy resources, after maintenance demands were covered (NS-2 and NS-3), were stored as carbohydrates and lipids. In NS-3 mussels, the ration was high enough to allocate energy into new corporal structures.

5.2 Biomarkers

5.2.1 Bioaccumulation

Bioaccumulation data evidenced two different groups in all mussels according to the two nominal FLU doses offered. Mussels exposed to 3 µg/L accumulated between 7-15 µg/g dw whereas those exposed to 60 µg/L exhibited a FLU concentration between

160-220 µg/g dw. These FLU concentrations might be comparable with those registered after an important oil spill as it was the *Prestige* disaster near the Atlantic Spanish coast. Total PAHs (sum of parent 13 PAHs) after this spill was around 8 µg/g dw (Soriano *et al*., 2006) which decreased to 2 µg/g dw of total PAHs after 1 year of depuration (Viñas *et al*., 2009). Lower values, close to 2.5 µg/g of total PAHs, were observed after the *Aegean Sea* oil accident in a nearby area (Porte *et al*., 2000). Higher values, however, were observed after other disasters such as the *Sea Empress* oil spill occurring in Wales (UK) where PAHs content (sum of 18 parent PAHs) in mussel tissues were higher than 25 µg/g dw in many sites (Dyrynda *et al*., 2000), and even above 500 µg/g dw at sites close to the spill site (Law and Kelly, 2004). In summary, it can be considered that the whole range of FLU concentrations obtained in the present study after exposure (7-200 µg/g dw) are realistic concentrations of PAHs after an important oil spill.

Results from the present study showed different patterns of FLU bioaccumulation according to the mussel nutritive state. NS-2 mussels accumulated the highest FLU concentration in their tissues, being this effect more evident at low FLU exposure (Table 12). Considering that FLU exposure conditions were adjusted in such a way that both, FLU concentration in aquaria and FLU doses per mussel biomass, were the same for each mussel condition, a similar accumulation pattern should be expected. However, NS-3 mussels showed a lower FLU accumulation (more than half at Low concentration) than NS-2 mussels. Previous field studies with trace metals evidenced that high tissue weight (high condition indices) involves low accumulation (Mubiana *et al*., 2006; Richir and Gobert, 2014; Zhong *et al*., 2013). This relationship was explained by the dilution effect associated with the high growth rates of high-condition mussels. In the field, mussels are exposed to the same pollutant concentration although the dose of pollutant per unit of mussel biomass would be lower in higher condition-mussels. On the contrary, in the present study, concentration and weight-relative dose was kept constant, which makes an alternative explanation needed. We think that this lower bioaccumulation registered in NS-3 mussels could be attributed to their lower food absorption rates. As mentioned in section 2.3., daily FLU doses were supplied with the same amount of microalgae for all treatments, which was completely filtered by all the mussels in a short time. Moreover, FLU water concentrations (data not shown) almost disappeared in 2 hours after FLU addition, what means that complete FLU daily dose was being incorporated by the ingestion of the FLU-adsorbed-microalgae cells. Regarding food absorption, NS-3 mussels processed food during the experiment (conditioning and exposure) with a lower AE, almost half than NS-2 mussels as was described in section 3.1. (Figure 24). If a higher portion of the FLU-incubated microalgae cells were passing through the digestive system without being absorbed in NS-3 mussels, it could be possible that, in these mussels, a higher portion of FLU was eliminated into the faeces which could explain their lower accumulation rates.

Following the above explanation, NS-1 mussels which evidenced the lowest CI, should show the highest accumulation values, but these values were significantly lower than those found for NS-2 mussels at low FLU concentration. Organic pollutants are known to accumulate in lysosomes which are present in high numbers in tissues such as the digestive gland (Moore, 2008). Under NS-1 condition, autophagy is induced which

could promote a quicker detoxification for the nutrient deprived cells and, as proposed by several authors, facilitating non-specific detoxification of cytosolic PAH at a greater rate than in the fed PAH-treated cells (McVeigh *et al*. 2006; Moore *et al*., 2006, 2007).

To conclude, one of the most basic assumptions in environmental biomonitoring that sentinel species accumulate pollutants in direct proportion to pollutant environmental concentrations (Goldberg, 1986) is clearly compromised in view of the present results. Our results indicate that some biological factors as food absorption efficiency or autophagic induction can affect bioaccumulation.

5.2.2 Physiological biomarkers

Our experimental results showed a clear inverse relationship between CR (measured under the same food concentration) and mussel nutritive state as it was previously described in our field studies (Albentosa *et al*., 2012a, Bellas *et al*. 2014, González-Fernández *et al*., 2015). According to Filgueira *et al*. (2008), the effect of condition on the CR is evident when CRs are standardized to weight, which is the most usual standardization. The length standardization ($CR_{L\text{-}st}$) is recommended though (Filgueira *et al*., 2008) as the size of the filtering organ (gill size) is more related to shell length than to corporal weight, due to changes in body mass unrelated to gill size, but related to reproduction or nutrition (Crandford *et al*., 2011). However, in our experiment, although mussel shells length was the same for all treatments, significant differences were detected in gill size depending on nutrition (Table 13). Therefore, CR was standardized to gill size ($CR_{Gill\text{-}st}$). $CR_{Gill\text{-}st}$ was 3 times higher in low food mussels than in those fed at the highest ratio (Table 13). The functional response of bivalves' CR to the variation in the seston concentration has been described by many authors (Winter, 1978; Widdows, 1978; Bayne and Newell, 1983; Albentosa *et al*., 1996; Hawkins *et al*., 2001; Velasco and Navarro, 2003) and consists in the decrease of clearance rates as seston concentration increases over a narrow to broad range. Similar feeding behavior was observed in the present study, where higher CRs were observed in the low-feeding mussels which were kept at low food concentration for a period of two months. The fact that mussels showed different CR depending on their condition, despite that CR measurements were done under the same food concentration, suggests that mussels have an "ecological memory" that comes from their past-feeding history. Moreover, this "memory" remains for more than the 24h that mussels were in the open-flow system, before the CR measurements were carried out. A period of acclimation of 24h is the time period established by ICES to perform SFG test in monitoring programs (Widdows and Staff, 2006). The concept of "ecological memory" was initially proposed by Mallet *et al*. (1987) and later used by other authors (Bayne 1993; Pérez-Camacho *et al*., 1995; Iglesias *et al*., 1996; Labarta *et al*. 1997; Babarro *et al*. 2000; Fernández-Reiriz *et al*., 2013). In these studies, mussels from different origins (subtidal/intertidal areas) or from reciprocally transplanted experiments, showed marked physiological differences when placed under the same ambient, and those differences would persist for several weeks (Babarro *et al*., 2000) or months (Widdows *et al*., 1984). These differences might be related to physiological adaptations to the particular environmental

conditions in the primary habitat (Babarro *et al*., 2000) or to genetic differentiation (Peterson and Beal, 1989). In the present experiment, where all mussels came from the same site, the physiological adaptation to food conditions before the SFG test seems to be the explanation of the differences observed in CRs.

Regarding the effect of FLU on CR, previous studies have reported that CR is the physiological component that is most affected by pollution (Al-Subiai *et al*., 2012; Beiras *et al*., 2012; Howell *et al*., 1984; Eertman *et al*., 1995; Toro *et al*., 2003, and references therein). Similarly to previous studies where mussels exposed to organic pollutants evidenced a decrease in CR with pollution (Donkin *et al*.,1989; Fernández *et al*.,2010; Widdows *et al*., 1987; Widdows and Jonhson, 1988), our results showed a decrease of CR with FLU exposure but only at Low FLU concentration. Moreover, this effect of FLU on CR was only evidenced in NS-2 mussels, that could be related to the higher FLU accumulation in their tissues (accumulation efficiency close to 12%, almost double than in the other two conditions). Since the FLU effect is only evident for mussels with intermediate nutritive state, we suggest that the other two conditions confer mussels more resistance to pollution. NS-1 mussels which are under nutritive stress seem to be resistant to FLU exposure as mentioned in the literature (Moore, 2004; Moore *et al*., 2007). Apart from NS-2 mussels exposed at Low concentration, CRs of exposed mussels were similar to controls (Table 13). This result is supported by Toro *et al*., (2003) who emphasized that some PAHs as fluoranthene or pyrene can be accumulated in large quantities on mussels with little or no effect in CR. Moreover, tolerance to PAHs concentration in tissue may explain the lack of physiological effect in mussels (Thomas *et al*., 1999). Other studies have found similar result when mussels were exposed to metals (Tsangaris *et al*., 2008). Anyhow, according to F-test from the two-way ANOVA (Figure 28), the effect of mussel nutritive state (F_{NS}≈40) was 10-fold higher than the effect of toxicant (F_T≈ 3), which could mask FLU effects.

In terms of absorption efficiency (AE), it is necessary to differentiate between mussel absorption in conditioning/exposure period (Figure 24) and the results obtained in the SFG test (Table 13). During the conditioning/exposure period, mussels fed with higher rations showed lower absorption efficiencies than mussels fed with lower rations. This process has been previously reported in bivalves (Albentosa *et al*., 1996; Bayne and Newell., 1983; Beiras *et al*., 1994) and consists in the decrease of absorption efficiency as particle concentration, and consequently ingestion (IR) increases. This inverse relationship between AE and IR is explained by the gut passage time which decreases as IR increases (Gosling, 2003). When mussels were maintained under the same food concentration as was the case of the SFG test, the contrary results were obtained with higher AE in NS-3 mussels. This contradiction was only apparent, as AE in the SFG test presented the same pattern as during conditioning/exposure period: lower AE as IR increases. Indeed, lower values of AE were observed at NS-1 mussels which showed the highest IR during the SFG measurements. It seems, therefore, that for AE measurements, a period of 24 h before faeces sampling, as applied in the present experiment, is enough to depurate the old faeces produced by the aquaria feeding conditions. An alternative, and probably complementary, explanation of the higher AE

registered in NS-3 mussels might be their larger enzymatic equipment (although less efficient) that is supposed in well-feeding mussels.

In spite of the above-mentioned effect of mussel nutritive state on AE, it was considerably smaller (F_{NS}≈5) than that of FLU exposure (F_T≈30). Moreover, FLU effect on AE was dependent on the nutritive mussel condition (F_{R*T}≈15). Although higher AEs were observed in mussels exposed to low FLU at NS-1 and NS-2 nutritive condition, the most pronounced effect of FLU on AE was observed at the NS-3 food condition, with more than 50% AE decrease at the high FLU concentration, during the SFG assay. In mussels, many authors have reported that PAHs exposure can induce pathologic changes in digestive the gland and lysosomal membrane stability (Lowe *et al*., 1981; Toro *et al*., 2003, Porte *et al*., 2001a; Solé *et al*., 1995). Studies on *Crassostrea virginica* (Weinstein, 1997; Al-Subiai *et al*., 2012) evidenced that exposure to 100 µg/L FLU caused a reduction in their mean digestive epithelial thickness. Similar results were described by Jeong and Cho (2007) in *C. gigas* exposed to 200 µg/L, which decreased the absorption efficiency from 3 weeks until the end of the experiment. Histological changes in the digestive tubules could thus be responsible of the poorer functioning of the digestive gland in the NS-3 mussels. In spite of the AE decrease in the high FLU-exposed mussels during the SFG test, no differences were observed among treatments in the NS-3 mussels during AE estimation in the acclimation/exposure period (Figure 24). The coexistence of two powerful stressors as nutritive and pollutant stress on physiology, makes the identification of the physiological response to pollution really complex.

Respiration rates (RRs) were also more affected by mussel nutritive state (F_{NS}≈13) than by FLU exposure (F_T≈40). The strong differences observed in mussels' RRs might be related to standardization, which considers respiration rates per mussel dry weight, as in our experiment, mussel dry weight differed greatly between conditions, being NS-3 close to 30% and 50% higher than NS-2 and NS-1 conditions, respectively. If we consider RR per individual, no significant differences were observed between NS-1 and NS-2 conditions, although both are still higher than NS-3 (data not shown). In a similar laboratory experiment where mussels were conditioned at four food ratios (Albentosa *et al*., 2012b), lower RRs were reported under low food conditions, contrary to the results of the present study. It has been widely described that bivalves reduce their metabolic costs in response to low particulate concentration as a functional response to reduce energy losses (Newell and Branch, 1980; Widdows and Hawkins, 1989). In these studies, metabolic costs were measured at these low-food environments. On the contrary, in the present work, RRs in NS-1 mussels, for instance, were measured after 24 h of high-food conditions such as those applied in the SFG test (1.3 mg/L of total particulate matter). Under these favorable food conditions, high CRs and consequently high IRs are registered in NS-1 mussels which require higher metabolic costs. These metabolic costs associated with an increase in feeding activity include, not only the energy used for filtration, but also the costs to process and digest of ingested food (Griffiths and Griffiths, 1987). The shift between the low-food conditions in NS-1 aquaria to the high-food conditions in the SFG test should induce the synthesis of

digestive enzymes, which could be responsible for the highest metabolic costs in NS-1 mussels.

With regard to the toxicant, mussel RR from NS-2 and NS-3 conditions were also affected by FLU exposure. The increase in the energy lost through respiration after toxicant exposure has been previously reported by several authors (Widdows *et al*., 1982; Donkin *et al*., 1989; Widdows and Donkin., 1991; Toro *et al*., 2003b). Several causes have been suggested for the increase of oxygen consumption in bivalves exposed to hydrocarbons (Jeong and Cho, 2007): uncoupling of oxidative phosphorylation (Bayne *et al*., 1982), enzymatic activity for the degradation of petroleum components (Stekoll *et al*., 1980) and increase in the activity of phosphofructokinase in glycolytic pathways in the gills and digestive gland (Widdows *et al*., 1982). As previously pointed out, NS-1 mussels do not seem to be affected by FLU exposure, probably for the protection offered by autophagy processes (Moore *et al*., 2004; Moore *et al*., 2007). According to Pipe (1987), the reallocation of energy reserves from reabsorbed oocytes in *M. edulis* following exposure to petroleum hydrocarbons may serve as a resistance strategy to survive the effects of hydrocarbon exposure. The catabolism of stored energy could be responsible for the increased RR in exposed mussels and could also justify the decrease in CH content observed at High exposure in the three mussel conditions (Table 11).

As SFG results from the integration of the above-mentioned physiological rates into the energy balance equation, the combined effect of the considered factors, nutritive state and FLU exposure, reflects what was said above: a strong effect of mussel state and an almost non-existent effect of FLU exposure on SFG ($F_{NS}\approx57$ and $F_{T}\approx1$, for condition and toxicant, respectively, Figure 28). Thus, the highest SFG value was observed in NS-1 mussels being almost 2-fold of that observed in NS-2 mussels and more than 5-fold the SFG of NS-3 mussels. Although SFG depends on the difference between the energy acquired and the energy lost in metabolic costs, it has been widely recognized that absorption is the physiological rate with the greatest effect on the SFG estimation (Widdows, 1985). Absorption depends on both, the feeding rate and the efficiency of food processing. As mussel state slightly affected AE (Figure 25), the main effect of mussel condition on the SFG was its effect on CR. Therefore, the SFG response clearly evidenced the effect of mussel state on feeding. Moreover, the higher RRs observed (metabolic costs) at lower mussel condition did not change the correlation of SFG with mussel state. The great effect of nutritive state on feeding was high enough to counteract the negative effect of metabolic costs on SFG estimation.

The strong effect of mussel condition on feeding rates measured during the SFG assay, provides a serious problem for the use of this biomarker in field monitoring programs, but validates the hypothesis previously suggested by our group in field studies that SFG was negatively correlated with mussel condition. In the present study, the same relationship was observed: $r = -0.61$, $R^2 = 37.4\%$, $p<0.001$. Here, we induced mussels to obtain a wide range of CI manipulating food conditions but the range of CI obtained: 5.2 (±0.6) to 8.9 (±1.9) was not as high as that observed in our annual monitoring studies where mussel CI ranged from 8.7 (±1.3) to 14.1 (±1.3) in 2012 (González-Fernández *et al*., 2015) or 6.8 and 7.8 to 13.5 and 18.2 in 2007 and 2008

respectively (Albentosa *et al*., 2012a). These strong differences in mussel condition were not only observed between sites, but also between years for the same site, as was evidenced in Albentosa *et al*., (2012a), hindering the interpretation of physiological biomarker responses in large-scale-monitoring programs.

More surprisingly, SFG values were not affected by FLU exposure at NS-2 and NS-3 mussel state whereas at NS-1 condition there was only a weak effect of exposure. In this last case, the effect was contrary to expected, as SFG was higher in exposed mussels. Since the 1980's, SFG measurements have been used in the assessment of the toxicity of pollutants such as hydrocarbons both, under field (Widdows *et al*., 1995; 2002) and laboratory (Widdows *et al*., 1982; 1984; Donkin *et al*., 1989; Widdows and Donkin, 1991; Eertman *et al*., 1995) conditions. In these studies, low or even negative values of SFG were related to PAHs accumulation in mussels. On the contrary, we have never found any significant correlation between SFG and PAHs concentration in mussel tissues within the Spanish annual pollution monitoring program (Albentosa *et al*., 2012a; Bellas et a., 2014; Fernández-González *et al*., 2015). Similar results were described by Thomas *et al*. (1999) or Wang *et al*. (2005), whom did not observe any significant relationship between SFG and PAHs levels. In agreement with the conclusions of our previous work, these authors explain SFG differences in terms of the important differences registered in environmental factors. The present study demonstrates that SFG measurements are highly dependent on mussel nutritive state, which hampers the use of this biomarker in the assessment of environmental pollution.

In addition, we found a more significant effect of FLU exposure than mussel state on one of the physiological processes measured, the absorption efficiency, but the effect on this variable is hidden once integrated in the SFG due to the higher effect of condition in feeding. For that reason, we recommend to analyze each individual component of the energy balance equation before their integration in the SFG estimation. Moreover, each stressor may have a specific effect in one of the physiological rates included in the SFG estimation but not in another, as they are controlled by different biological functions.

5.2.3 Biochemical biomarkers

In this study, we observed that NS-1 mussels showed the highest antioxidant responses (SOD, CAT, GPx and GST), that means an inverse relationship between the antioxidant responses and mussel nutritive state, as it was previously suggested in field studies by our research group (Bellas *et al*., 2014; González-Fernández *et al*., 2015). Other studies described similar results in limpets (Ansaldo *et al*., 2007) and in fish (Martínez-Alvarez *et al*., 2005; Morales *et al*., 2004; Pascual *et al*., 2003). Although low food availability decreases metabolic activities, it also causes degradation of endogenous biochemical components as proteins, glycogen and lipids in order to obtain the energy required by the maintenance metabolism (Ansaldo *et al*., 2007). As commented above, under these conditions of dietary restriction, tissue autophagy is induced by the increase of lysosomal number and activity (Moore, 2004; Moore *et al*., 2007) which are sites of ROS generation (Moore *et al*., 2007) and, as a consequence,

antioxidant defense systems could be activated. In present study, SOD, CAT, and GPx activities were affected by mussel nutritive state, which might be a consequence of the increase of ROS caused by the biochemical components consumption process in NS-1 mussels, compared with the other two conditions, in agreement with Morales *et al*. (2004). Several authors have described seasonal variability of antioxidant enzymes as CAT or GPx, which could be related to the above mentioned influence on the food availability on the antioxidant defenses (Livingstone, 2001; Botichelli and Regoli, 2006; Vidal-Liñán *et al*., 2010). In addition, GST activity was also affected by mussel nutritive state in a similar way as the antioxidant enzymatic activities, as previously reported by Ansaldo *et al*. (2007) in starved limpets. In agreement with our results, Leiniö and Lehtonen, (2005) observed changes in *M. edulis* GST activity in correlation to seasonal patterns in food availability. GST enzymes play an important role in the antioxidant system, since organic hydroperoxides, alkenals and epoxides resulting from oxidative metabolism may be regarded as 'natural' substrates for this enzyme. Therefore, the high levels of GST detected in NS-1 compared to NS-2 and NS-3 may suggest an increase in the utilization of GSH in conjugation reactions involved in the metabolism of biochemical endogenous components and/or products derived from the oxidative metabolism.

In summary, it seems that stressed-fed mussels (NS-1) were more resistant to FLU effect, caused by the activation of the autophagic system under these adverse feeding conditions, acting as a mechanism for antioxidant protection (Moore *et al*., 2007). This involves the removal of oxidative damaged proteins, impaired organelles and even portions of the nucleus and DNA (Moore *et al*., 2008).

As it can be seen from Figure 28, the F test shows that the effect of mussel nutritive state was 7-fold higher than the effect of toxicant for SOD and GPx activities and almost 40-fold higher for CAT. Thus, variability of antioxidant activities related to endogenous factors as nutritive state, could mask pollution effects as was widely described (Sheehan and Power, 1999; Leiniö and Lehtonen, 2005; Gagné *et al*. 2008).

In contrast to the previous antioxidant biomarkers, GR activity did not show statistically significant differences between nutritive conditions. This enzyme catalyses the reduction of oxidized glutathione (GSSH) to GSH, therefore it is not directly related to ROS production induced by the autophagy which is supposed for NS-1 mussels. Thus, this enzyme was not affected by mussel nutrition as were the other antioxidant enzymes, which mediated in the neutralization of these ROS species (CAT, SOD, GPx or GST). Moreover, GR is responsible for the maintenance of the correct GSH/GSSG ratio and the intracellular redox state in marine organisms (Regoli and Giuliani, 2013), that is to say one of the most essential cellular processes which could be later (after failure of other antioxidant defenses) affected if stressful conditions continue or intensify.

It is well known that pollutants, and specifically PAHs (Solé *et al*., 1995; Porte el al., 2001a; Fernandez *et al*., 2010), increase the ROS production in mollusks and the antioxidant defenses in response to this ROS enhancement (Viarengo *et al*., 1990; Winston and Di Giulio *et al*., 1991; Manduzio *et al*., 2005; Rodrigues *et al*., 2013). Moreover, some of these studies indicated that the antioxidant enzymes can increase

their activity at low concentrations of pollutants, but can be inhibited at high concentrations (Livingstone *et al*., 1990; Porte *et al*., 1991; Regoli and Principato, 1995), which is in agreement with our results. In this study, mussels under nutritive stress (NS-1) were protected by high levels of antioxidant enzymes induced by the nutritive stress, thus the increase of ROS production associated to the exposure to FLU, was not evident as the antioxidant activity levels have already increased. Contrary, in mussels with higher food conditions (NS-2 and NS-3), the levels of antioxidant biomarkers (CAT, GR and GPx) were higher in mussels exposed to low FLU exposure than controls. This is the usual pattern of biomarker responses, increasing with higher ROS concentrations, in agreement with Cheung *et al*. (2001), who observed higher levels of GR, GPx and SOD activity in mussel hepatopancreas in the presence of benzo[a]pyrene, and with other studies reporting the induction of CAT in mussels exposed to PAHs (Eertman *et al*., 1995; Fernández *et al*., 2010; Porte *et al*., 1991; Sureda *et al*., 2011). According to the present study, the described antioxidant responses in these studies may have been completely different if other nutritive conditions had been applied. Therefore, the variability of antioxidant activities related to nutritive state could mask pollution effects in field studies.

It should be expected that the effect of FLU exposure on GR would be more evident than in the other antioxidant activities, as this enzyme was not affected by the mussel nutritive condition. However, GR appears to be more sensitive to metals exposure than to PAHs, both in gills (Fernández *et al*., 2010) and in digestive glands (González-Fernández *et al*., 2015).

Finally, the increase of antioxidant activities was not observed at high concentration, which may be associated to toxic effect on these enzymes. This fact has been described in other studies in clams (Liu *et al*., 2014) and scallops (Pan *et al*., 2005), where low levels of PAHs exposure led to the induction of GST, while high level of PAHs exposure resulted in the depression of GST. This was explained by an inhibitory effect caused by high pollutant concentration in tissues, or as an adaptive response where mussels may change the GST detoxification pathway in favor of a different detoxification response.

Contrary to the rest of biomarkers, the levels of LPO detected in NS-1 were similar to those found in NS-2 and NS-3 mussels, which may indicate that antioxidant defenses were able to avoid the damage of cellular components, such as lipids, under the nutritive-stressful conditions at NS-1. In previous studies, the increase in the antioxidant enzymes activity in polluted areas was accompanied by no significant differences in MDA concentration, showing an adaptation to the contaminant exposure (Box *et al*., 2007, Cheung *et al*., 2001), or even an inverse relationship between antioxidant responses and LPO, suggesting a protector role of antioxidant biomarkers (Campillo *et al*., 2013; Fernández *et al*., 2010; González-Fernández *et al*., 2015).

5.3 Integrative assessment

All biomarker responses were integrated in a PCA analyses in order to obtain a general picture of biomarker responses to nutritive state and toxicant. All biomarkers studied, apart from AE and LPO, were included in the first two components of the PCA analysis which explained more than 60% of the variance. PC1 clearly separates mussel groups according to their nutritive condition (Figure 26). NS-1 mussels were characterized by high SOD, CAT, GPx, CRs and RRs levels, independently of the FLU exposure. On the contrary, low levels on these biomarkers were observed in NS-3 mussels whereas in NS-2 mussel groups intermediate levels were measured. PC2 brings together mussels exposed to low FLU concentration in NS-2 and NS-3 mussel state and to control and high FLU concentration in NS-1 mussels. This grouping was based on high GST and GR. It seems that both activities were more affected by toxicant exposure than nutritive state, contrary to the biomarkers grouped by PC1, although this effect was only evident at low FLU concentration. In spite of this result, the variance explained by PC1 was double than that explained by PC2.

Thus, all biomarkers measured, including several levels of biological organization, both biochemical and physiological, showed the same pattern. Biomarker responses were more affected by mussel nutritive state than by toxic exposure. This conclusion should not be a surprise, as food availability, selected as the determining variable of mussel condition, had long been identified as one of the most relevant environmental factors affecting biological processes. Mussels are intertidal organisms exposed to extreme high food fluctuations in both, quality and quantity. Food regimes in estuarine habitats are subjected to changes in time in the short-term, such as the changes associated to tidal regimes or, in the long-term, to seasonal changes. As a mechanism of adaptation to these variable environments, marine bivalves exhibit a high flexibility in their morphological (Honkoop *et al*., 2003) and physiological traits (Ibarrola *et al*., 1996, 1998a, b). Moreover, estuarine ecosystems and especially near-shore coastal areas where mussels inhabit are subjected to a high anthropogenic pressure. Thus, biological responses of animals from these complex environments are the integrated result of all factors that affect them. The variety of biotic and abiotic factors that can modify biological responses, the compensatory mechanisms of biota under environmental changes, or the complexity of biological systems, make the establishment of cause-effect relationships between stressors and biomarkers quite difficult (Adams, 2005).

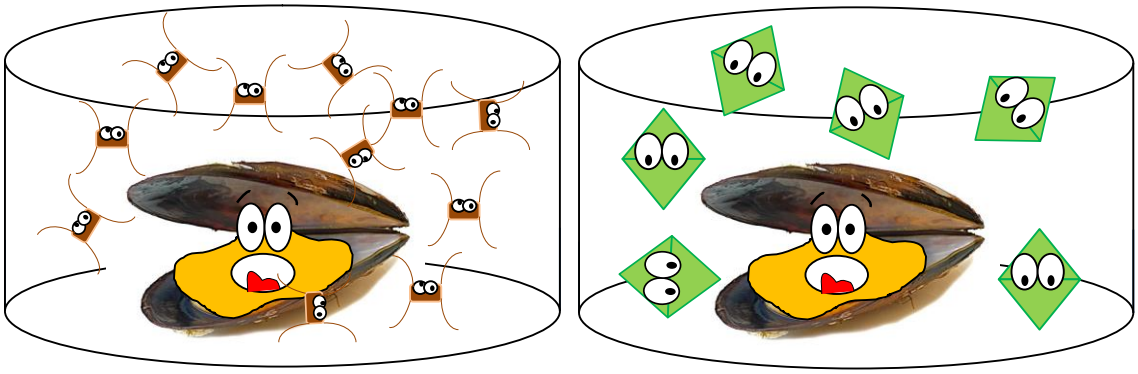
Furthermore, the reproductive state is closely related to food availability and temperature (Bayne, 1976; Gabbott, 1975). Thus, both mussel condition and reproductive state are closely associated, and it is impossible to separate the effect of internal (gametogenesis) or external (trophic conditions) factors on related biomarker responses. In large-scale monitoring programmes where both, internal and external conditions, are interacting at the same time , it is even more difficult to interpret the effect of pollutants on biomarker responses, as was illustrated in González-Fernández *et al*. (2015). For that reason, laboratory experiments providing controlled conditions are essential to assess the biological effect of pollutants under different environmental scenarios and allowing the correct interpretation of data obtained in field studies. Here,

an environmental variable (food availability) and another external variable (pollutant) were tested, whilst internal variable (reproductive state) present in field studies was eliminated. In fact, at the end of the experiment, all mussel condition groups were in the same reproductive state (not ripe), which allows us to have a better comprehension of the biological effects on biomarkers.

Marine pollution monitoring programs aim to establish the link between chemical pollution and harmful effects on marine organisms. This requires the development of background levels and assessment criteria for both pollutants and biological responses, which provide the scientific basis to design measures to improve environmental quality. However, this paper demonstrates that toxicants are not the only source of variability modulating biological responses to pollution, and confirms nutritive state as a major factor altering biochemical and physiological activities. Therefore, trophic conditions should be considered, integrating this information with that of chemical analyses and biological responses to better understand the variance of the data, particularly in large-scale monitoring programs covering a wide range of environmental scenarios.

CHAPTER 3

Condition II: Nutritive state-Quality



Carmen González Fernández, 2016

The research presented in this chapter forms part of the following publication:

González-Fernández, C., Lacroix, C., Paul-Pont, I., Le Grand, F., Albentosa, M., Bellas, J., Viñas, L., Campillo, J.A., Hégaret, H., Soudant, P. 2016. Effect of diet quality on mussel biomarker responses to pollutants. Aquat. Toxicol_XX

Journal category: Marine & Freshwater Biology
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IF: 3.948
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ABSTRACT

The main objective of this study was to assess the effect of primary production quality on some biological and biochemical responses used as indicators of pollution. For that purpose, mussels were conditioned for 6 weeks with the diatom *Chaetoceros neogracile*, and the dinoflagellate *Heterocapsa triquetra*, selected as two well differentiated types of primary production quality. These two microalgae species differ in several aspects which include biometric and biochemical characteristics. Upon dietary conditioning, mussels were exposed to a PAH, fluoranthene (FLU), for 1 week. Results evidenced higher FLU accumulation in mussels fed with *C. neogracile* (*C*-mussels) compared to those fed with *H. triquetra*. Concomitantly, greater effects of toxicant were observed in the biomarker responses of mussels fed with *C*-mussels. These mussels showed an increase of percentage of dead hemocytes, as well as an activation of phagocytosis capacity and ROS production after exposure. Some enzymatic activities were also increased upon FLU exposure (SOD, CAT, and GR). Not only FLU exposure but also food quality influence biomarker responses, showing higher values of SOD, CAT and GR non-exposed *C*-mussels. In addition, for the same FLU concentration, GR response upon exposure varied according onto dietary conditioning, suggesting that diet could act as a confounding factor in biomarker responses to pollution. Consequently, trophic conditions should be considered in marine pollution monitoring programs for a better interpretation of biomarker responses.

Keywords: Food quality, mussels, biomarkers, monitoring programs, Fluoranthene

1. Hypothesis

In Chapter 2, the effect of food availability was evidenced on mussel biomarker responses, being this effect even higher that effect of toxicant. Not only food availability but also food quality is strongly variable in natural marine environments. In a similar way as in previous chapter, the present hypothesis is that mussel pollution biomarkers are affected by the quality of food available in the marine environment.

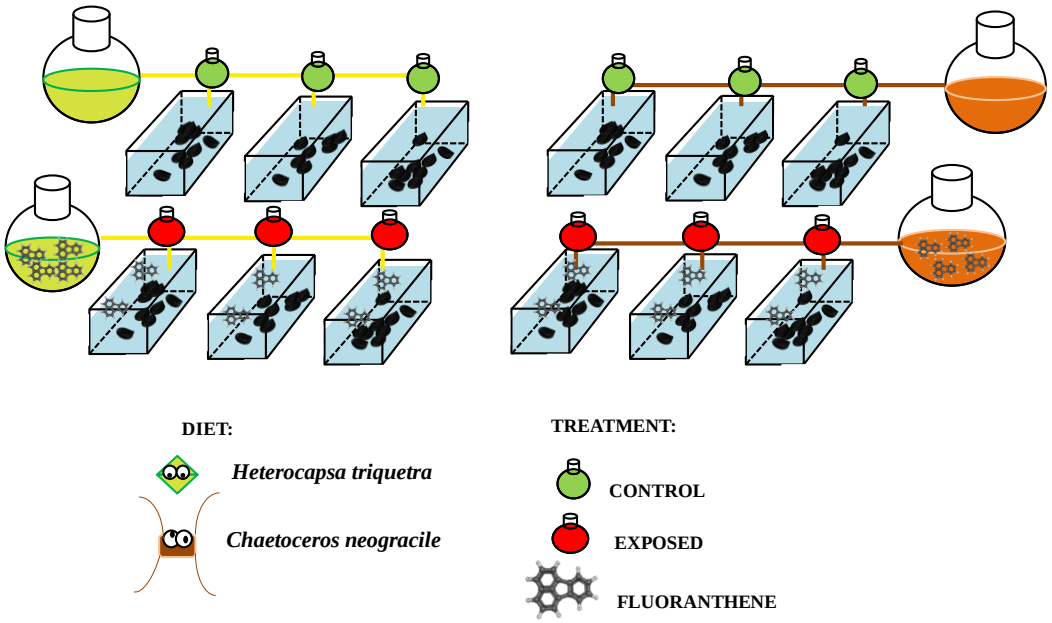
2. Objective

The overall objective of this chapter was to assess, under laboratory conditions, the responses of different biomarkers to pollution of mussels with different quality nutritive conditions.

The specific objectives of this chapter were to identify i) the effect of food quality on a battery of immunological and biochemical biomarkers, ii) the combined effect of pollutant and mussel dietary condition on those biomarker responses and, iii) the consequences of the obtained results in large-scale monitoring programs.

3. Summary of experimental design

Mussels were conditioned for 6 weeks to the diatom *Chaetoceros neogracile*, and the dinoflagellate *Heterocapsa triquetra*, selected as two well differentiated types of primary production quality. These two microalgae species differ in several aspects including biometric and biochemical characteristics. Upon dietary conditioning, mussels were exposed to a PAH, fluoranthene (FLU).



4. Results

4.1 Algae description

Algae description is shown in Table 16.

Table 16. Biometric and biochemical composition of microalgae dietary conditioning. Total nutrients supplied during exposure, expressed per individual and for the total exposure period (7 days), have also shown.

Diets	***Chaetoceros neogracile*** **C**	***Heterocapsa triquetra*** **H**
Phylum	*Bacillariophyta*	*Miozoa*
Class	*Bacillariophyceae*	*Dinophyceae*
Common name	diatom	dinoflagellate
	Microalgae biometrics	
Cell length (diameter) (µm)	4.1	16.2
Cell surface (µm²)	86.3	1077.8
Cell volume (µm^3)	91.2	3263.0
Cell wall composition	silica	cellulose
	Microalgae composition	
Cell dry weight (pg cell^{-1})	45.8	1465.6
Proteins (pg cell^{-1})	24.4*	827.1
Carbohydrates (pg cell^{-1})	6.1*	425.5
Lipids (pg cell^{-1})	15.1*	213.0
Total FA (pg cell^{-1})	0.23	3.67
PUFA (pg cell^{-1})	0.11	2.61
	Supplied during exposure	
µg FLU (ind)$^{-1}$	33.3	33.3
Total microalgae surface (cm²)	1329.0	519.7
Cells (ind) $^{-1}$	154×10^7	4.8×10^7
Total microalgae (mg ind^{-1})	70.5	70.7
Proteins (mg ind^{-1})	37.6	39.9
Carbohydrates (mg ind^{-1})	9.4	20.5
Lipids (mg ind^{-1})	14.1	10.3
Total FA (µg ind^{-1})	354.2	177.6
PUFA (µg ind $^{-1}$)	176.8	126.0

*data from Brown *et al.* 1997

Several parameters differ between algae species which included the biometrics and biochemical composition. The most evident difference is its size. *C.neogracile* consists of small cells which have 12-fold fewer surfaces than *H. triquetra*. Moreover, cell wall is also distinct between algae, being *C. neogracile* covered by a silica frustule whereas *H. triquetra* possess a cellulosic theca. Algae are, in addition, different in terms of biochemical composition. Total proteins, carbohydrates and lipids per cell was higher in *H. triquetra* being around 34, 70 and 14 fold higher respectively in comparison with *C. neogracile*. In order to feed mussels with the same algae ratio (1.5 % algae organic matter per mussel meat dry weight), a higher number of *C. neogracile* was added in *C*-mussel dietary conditioning. Despite this biomass adjustment, some differences of biochemical composition (as expressed as mg ind^{-1}) remained between diets, the diet *C. neogracile* supplied higher proportions of total lipids (a 50% higher) while the diet *H. triquetra* supplied higher content of carbohydrates (about 2-fold higher) (see Table 16 for more details).

Furthermore, looking at fatty acid (FA) levels (Table 17), statistical analysis evidenced significant differences in the main fatty acid groups between algae species. In general, higher proportions of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) were found in *C. neogracile* whereas higher proportions of polyunsaturated fatty acids (PUFA) were observed in *H. triquetra* ($p<0.05$; Student´s t test). Specifically, the main FA differences in both algae were determined by 18:5n-3, 16:3n-4, 20:5n-3, 16:1n-7, 18:4n-3, 16:2n-7, 22:6n-3, 14:0 and 18:3n-3, (57.9 % dissimilarity, SIMPER). Higher proportions of 14:0, 16:1n-7, 16:2n-7, 16:3n-4 and 20:5n-3 were observed in *C. neogracile* compared to *H. triquetra* ($p<0.05$, Student's-t test). A graphical presentation of algae according to their main fatty acid composition is shown in Figure 30A.

Table 17. Fatty acid composition of the two dietary conditionings: *C. neogracile* (diatoms) and *H. triquetra* (dinoflagellates) analyzed in the total lipid fraction (TL).

	Microalgae dietary conditioning	
Fraction	*C. neogracile*	*H. triquetra*
TMTD	0.1 [b]	0.0 [a]
BRANCHED	1.2 [b]	0.6 [a]
14:0	21.3 [b]	5.9 [a]
16:0	8.1 [a]	14.4 [b]
18:0	0.7 [a]	1.1 [a]
OTHER SFA	1.1 [b]	0.6 [a]
16:1n-9	0.1 [a]	2.4 [b]
16:1n-7	13.7 [b]	1.3 [a]
18:1n-9	0.2 [a]	0.7 [b]
18:1n-7	0.7 [a]	0.6 [a]
20:1n-11	0.0 [a]	0.0 [a]
20:1n-9	0.0 [a]	0.0 [a]
20:1n-7	0.1 [a]	0.1 [a]
OTHER MUFA	2.2 [b]	0.4 [a]
16:2n-7	6.1 [b]	0.0 [a]
16:2n-4	1.9 [b]	0.0 [a]
16:3n-4	13.0 [b]	0.3 [a]
18:2n-6	0.1 [a]	3.0 [b]
18:3n-3	0.0 [a]	2.2 [b]
18:4n-3	0.9 [a]	13.2 [b]
18:5n-3	0.1 [a]	32.2 [b]
20:4n-6	0.6 [b]	0.0 [a]
20:5n-3	19.8 [b]	1.1 [a]
22:2n-6	0.2 [a]	0.2 [a]
22:5n-3	0.0 [a]	0.3 [b]
22:6n-3	2.3 [a]	17.2 [b]
OTHER PUFA	5.0 [b]	1.7 [a]
NMI	0.0 [a]	0.0 [a]
DMA	0.0 [a]	0.0 [a]
UNKNOWN	0.5 [a]	0.8 [b]
SFA	31.3 [b]	22.0 [a]
MUFA	17.0 [b]	5.4 [a]
PUFA	49.9 [a]	71.2 [b]
TOTAL	100	100
PUI	1.89 [a]	3.42 [b]
PI	8.23 [a]	20.59 [b]

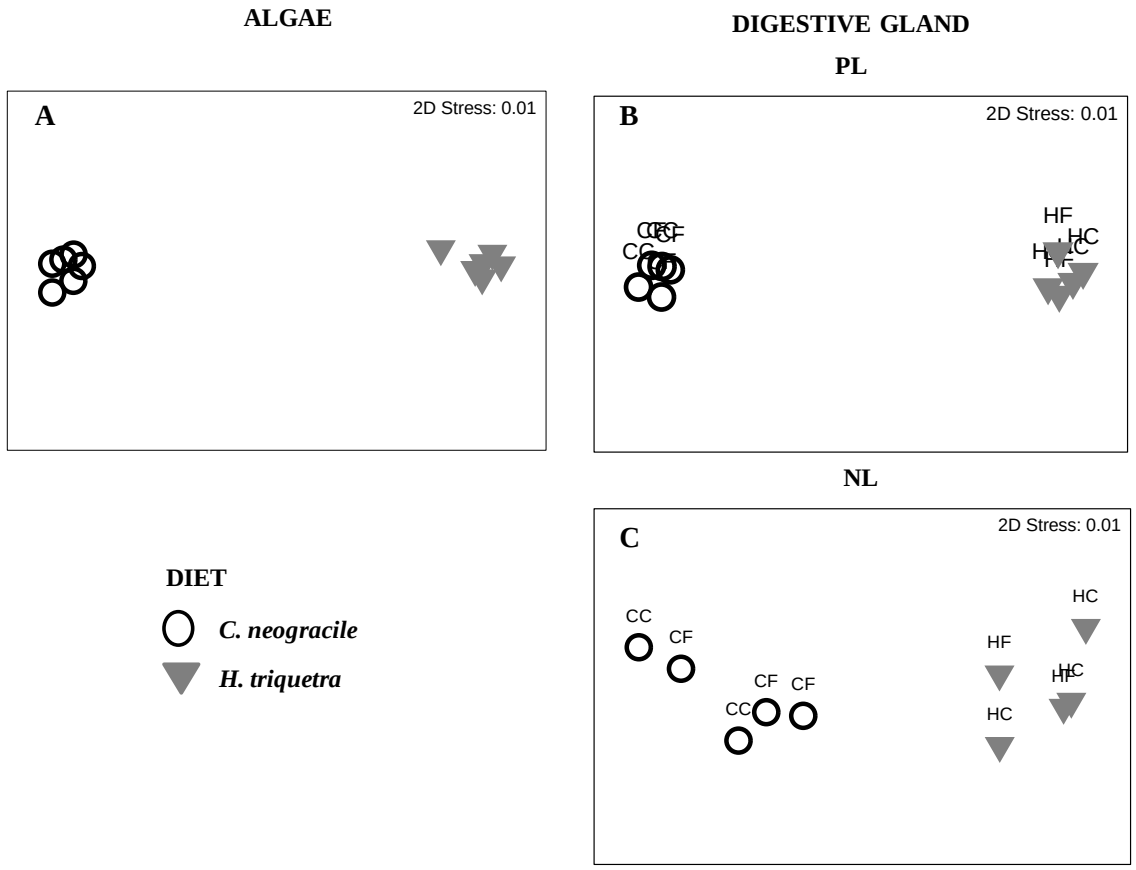


Figure 30. Non-metric multi-dimensional scale plots (NMDS) indicating the spatial distribution found in fatty acid composition (FA) analyzed on algae dietary conditioning and mussel´s digestive gland tissue. Figure A indicates spatial distribution of FA between dietary conditioning (*C. neogracile* or *H. triquetra).* Figure B and C indicate spatial distribution of FA in conditioned mussels (*C*-mussels or *H*-mussels) after 7 days of exposure to fluoranthene (FLU) and control in both lipid fractions, neutral lipids (B) and polar lipids (C). CC:Control mussels fed with *C. neogracile*, CF: FLU exposed mussels fed with *C. neogracile*, HC: Control mussels fed with *H. triquetra*, HF: FLU exposed mussels fed with *H. triquetra.* 2D-Stress levels <0.05correspond to an excellent useful representation.

4.2 Mussel biological characterization

4.2.1 Biological parameters

No significant differences were observed in mussel biological parameters between dietary conditions. Similar condition indices (CI) were observed in non-exposed *C*-mussels (9.6±3.5) compared to non-exposed *H*-mussels (8.2± 3.4). In the same way, for each dietary condition, no significant differences were observed between exposed and non-exposed mussels ($p>0.05$; Student's-t test). Exposed mussels showed values of 8.7±3.8 and 12.4 ±3.5 for *C*-mussels and exposed *H*-mussels, respectively.

Biochemical composition of mussels did not show significant differences between diets or between FLU treatments ($p>0.05$; Student's-t test). Non-exposed *C*-mussels showed values of 86.2±26.9, 204.0±32.6 and 709.7±57.0 mg g^{-1} AFDW of total

lipids, carbohydrates and proteins, respectively, whereas non-exposed *H*-mussels showed 93.2±8.5, 197.6±21.8 and 709.2±15.3 mg g^{-1} AFDW of total lipids, carbohydrates and proteins. In addition, exposed *C*-mussels showed values of 82.8±12.3, 151.5±24.3 and 765.6±17.6 mg g^{-1} AFDW, while exposed *H*-mussels evidenced values of 87.5±18.0, 218.5±41.5 and 693.9±29.4 mg g^{-1} AFDW, of total lipids, carbohydrates and proteins respectively.

4.2.2 Mussel fatty acid profiles

The NMDS plot (Figure 30B and 30C) showed the spatial distribution of mussel samples according to dietary FA imprint. A very good separation of mussel NL (stress=0.01, NMDS) as well as an excellent separation of mussel PL (stress=0.001, NMDS) was observed according to dietary conditioning.

According to the results of the SIMPER analysis, the main FA differences in digestive gland NL, considering the diet as difference factor, were determined by 18:4n-3, 16:2n-7, 16:3n-4, 14:0, 22:6n-3, 18:3n-3, 18:2n-6, 16:1n-7, 20:5n-3 (18.4 % dissimilarity, SIMPER). Higher values of 14:0, 16:1n-7, 16:2n-7, 16:3n-4, 20:5n-3 were observed in mussels fed with *C. neogracile* compared to mussels fed with *H. triquetra* ($p<0.01$, Student´s-t test). Dietary conditioning comparisons in mussel digestive gland for NL revealed significant differences between *C*-mussels and *H*-mussels (Global R: 1, Stress=0.001, ANOSIM). On the other hand, the main FA differences in digestive gland PL, considering the diet as difference factor, were determined by 18:4n-3, 16:2n-7, 14:0, 20:5n-3, 22:6n-3, 16:1n-7, 18:2n-6, 20:1n-9, 20:1n-11 (10% dissimilarity, SIMPER). Higher values of 16:1n-7, 16:2n-7, 20:5n-3 were observed in mussels fed with *C. neogracile* compared to mussels fed with *H. triquetra* ($p<0.01$, Student's-t). Dietary conditioning comparisons in mussel digestive gland for PL revealed significant differences between *C*-mussels and *H*-mussels (Global R: 1, Stress=0.001, ANOSIM).

No significant effect of FLU exposure was observed in digestive gland NL (Global R: 0, Stress=0.46, ANOSIM) or digestive gland PL (Global R:-0.04, Stress=0.59, ANOSIM).

4.3 Biomarkers

4.3.1 Bioaccumulation

Bioaccumulation data are shown in Table 18. All the mussels exposed to FLU showed significant higher FLU concentrations ($p<0.001$; Student-t test) with respect to non-exposed mussels considering both, digestive glands and whole tissues. Furthermore, this analysis evidenced that FLU accumulation in digestive gland and whole animal tissues (exposed mussels) was different according to dietary conditioning. *C*-mussel displayed higher FLU accumulation in their tissues.

Table 18. Fluoranthene (FLU) accumulation in whole mussel and digestive gland (µg g^{-1} tissue DW) after exposure time.

Diet	Treatment	Whole mussel	Digestive gland
H. triquetra	Control	1.32±0.37 ^{a}	0.80±0.33^{a}
	Fluoranthene	44.16±9.18^{bA}	55.41±29.19^{bA}
C. neogracile	Control	0.94±0.13^{a}	1.89±1.41^{a}
	Fluoranthene	106.74±7.87^{bB}	117.13±32.60^{bB}

Significant differences between exposure treatments (lowercase letters) and mussel diets (capital letters) were obtained from Student´s-t test at a significant level of $p<0.05$ (see text for details).

4.3.2 Immune parameters

Mean and standard deviation of immune parameters are shown in Figure 31.

ANCOVA analysis were performed on immune parameters and biochemical biomarkers in order to determine i) the effect of FLU exposure on these parameters (regression line fit), ii) the effect of diet without toxicant (intercept), and iii) the assessment of immune and biomarker responses to toxicant under different dietary conditioning (comparison between slopes) (Tables 19 and 20). Graphical representation of biomarkers affected by ANCOVA analysis is shown in Figure 32 to facilitate the understanding of effects reported below.

The effect of FLU concentration was observed in % of dead hemocytes ($p<0.05$; ANCOVA), phagocytosis capacity ($p<0.05$; ANCOVA) and hemocytes ROS production ($p<0.01$; ANCOVA). The % of dead hemocytes was significantly increased upon exposure to FLU in both *H*-mussels and *C*-mussels (Student-t test; $p<0.05$). However, the increases of phagocytic capacity ($p<0.01$) or ROS production with FLU exposure were only evidenced in *C*-mussels. Furthermore, a significant effect of dietary conditioning was observed in % of dead hemocytes, marked by a higher value in *H*-mussels (significant differences between intercepts $p<0.01$; ANCOVA).

Table 19. ANCOVA analysis of mussel immune parameters in relation with mussel FLU concentration, after 7 days of exposure to FLU.

Biomarker	Factor	DL	F	p-value
THC				
	FLU concentration	1	1.36	0.26
	Intercept	1	1.23	0.28
	Slopes	1	0.99	0.33
% of dead hemocytes				
	FLU concentration	1	6.45	**0.02**
	Intercept	1	16.04	**0.00**
	Slopes	1	0.07	0.80
Phagocytosis rate				
	FLU concentration	1	0.52	0.48
	Intercept	1	0.00	0.99
	Slopes	1	2.36	0.14
Phagocytosis capacity				
	FLU concentration	1	4.59	**0.05**
	Intercept	1	0.96	0.34
	Slopes	1	0.47	0.50
ROS production				
	FLU concentration	1	16.89	**0.00**
	Intercept	1	0.00	0.97
	Slopes	1	0.01	0.91

THC: total hemocytes concentration, ROS: Reactive oxygen species. Significant differences of ANCOVA at a significant level of $p<0.05$ are marked in bold.

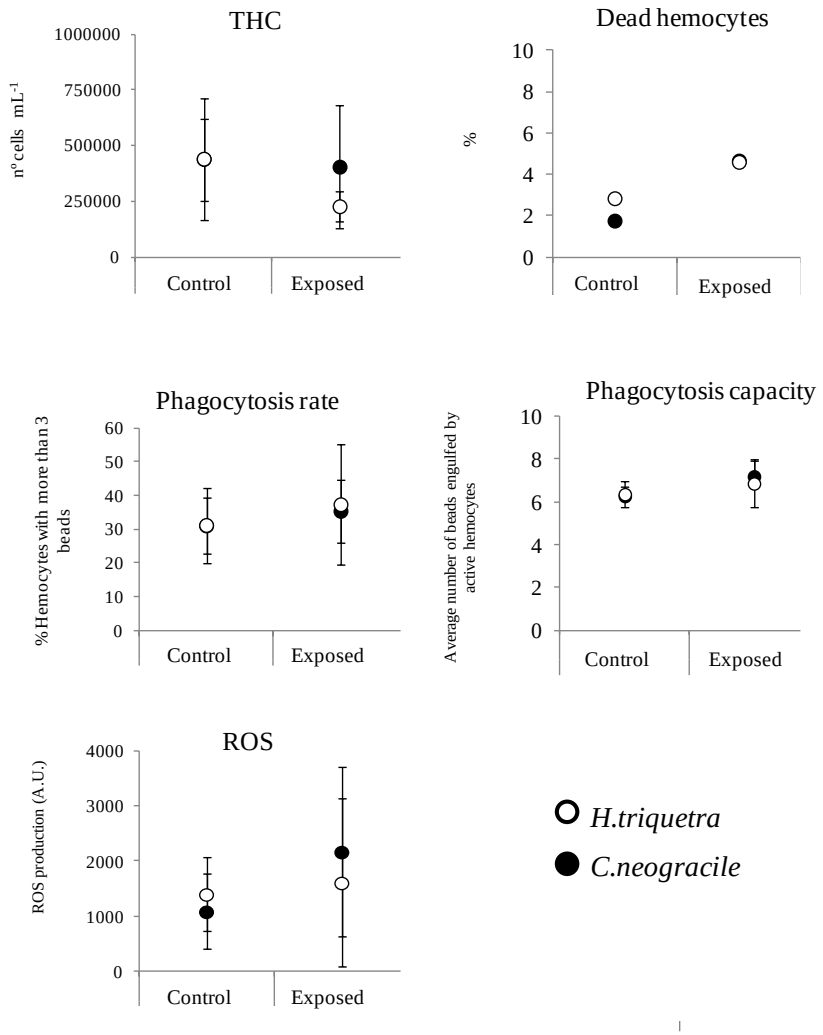


Figure 31. Summary of mussel immune parameters (means and standard deviations) dietary conditioned with *C. neogracile* or *H. triquetra* after 7 days of fluoranthene.THC: Total hemocyte count and ROS: Hemocyte reactive oxygen species production.

Graphical representation of immune parameters, significantly affected by FLU exposure in the ANCOVA analysis, is shown in Figure 32. This analysis did not reveal any significant effect in the slope for immune parameters which means that, immune responses to pollutants were similar in both dietary conditions. Therefore, dietary conditioning is not affecting mussels´ immune responses to FLU exposure.

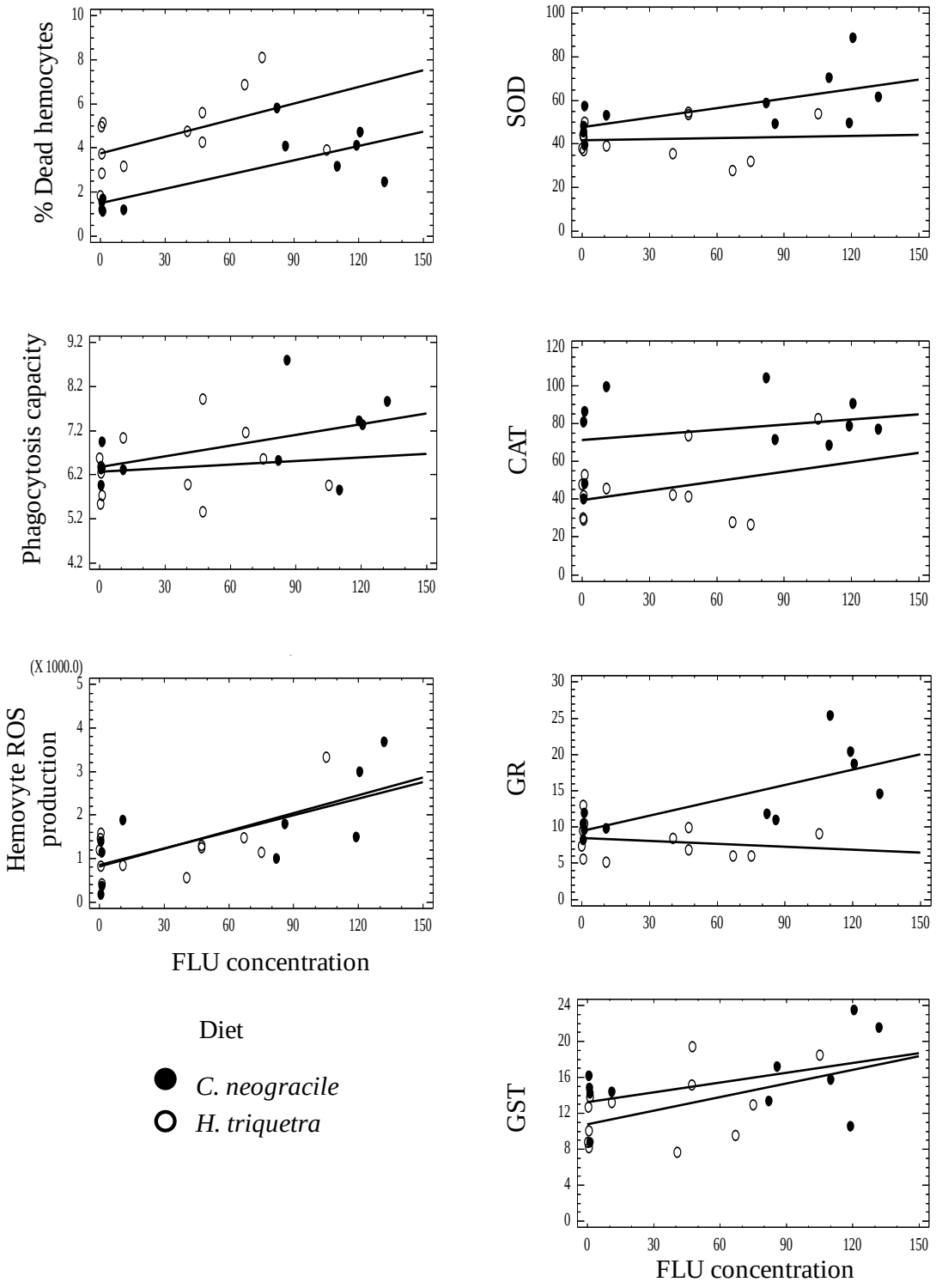


Figure 32. Graphical representation of immune and biochemical biomarkers with significant difference according to the diet and FLU exposure as tested by the analysis of covariance (ANCOVA) after exposure period. Mussel individual biomarker responses and individual fluoranthene (FLU) accumulation analyzed in mussel digestive glands were considered.

4.3.3 Biochemical biomarkers

Mean and standard deviations of biochemical biomarkers are shown in Figure 30.

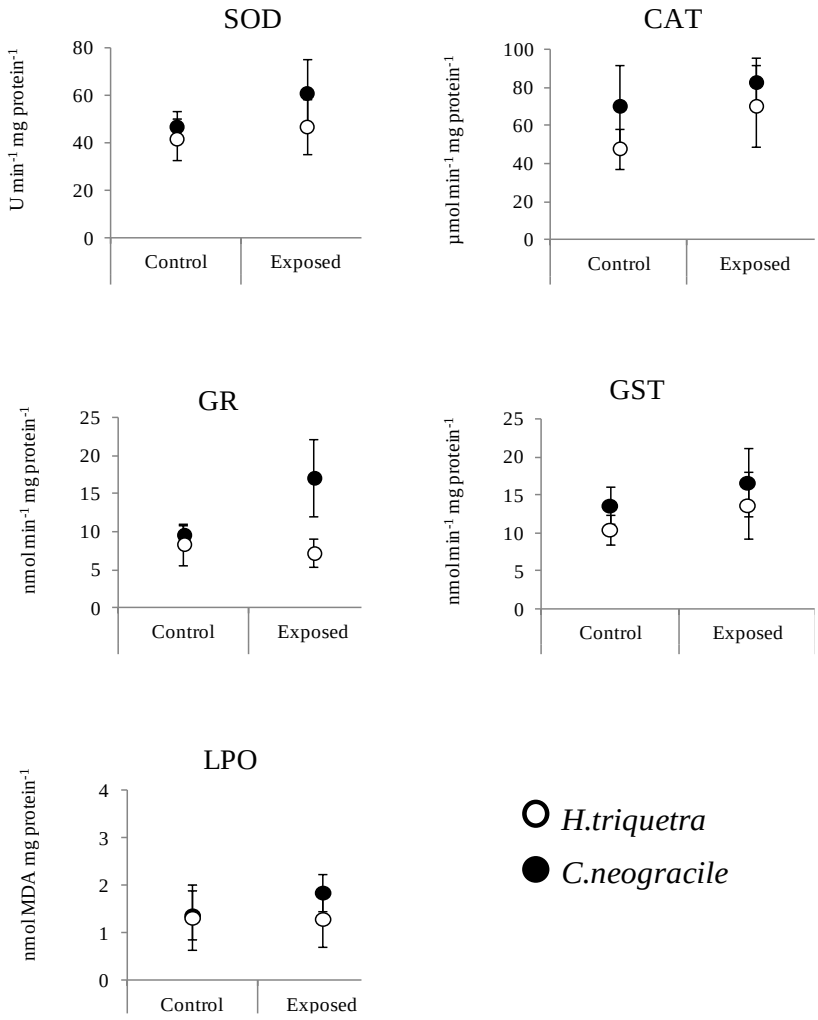


Figure 33. Summary of biochemical biomarkers (means and standard deviations) of mussels (*Mytilus spp.*) conditioned with two different diets after 7 days of FLU exposure. SOD: Superoxide-dismutase, CAT: Catalase, GR: Glutathione Reductase, GST: Glutathione-S-transferase, and LPO: Lipid Peroxidation.

The effect of FLU exposure was significant for all antioxidant enzymes studied (CAT, SOD, GR, and GST; $p<0.05$; ANCOVA, Table 20). Increases of SOD and GR activities upon FLU exposure were confirmed by the Student's-test in mussels fed *C. neogracile*. Moreover, a significant effect of dietary conditioning was observed in CAT ($p<0.01$; ANCOVA), SOD and GR ($p<0.05$; ANCOVA) marked by statistically significant intercepts. All three parameters were higher in *C*-mussels ($p<0.01$; Student´s-t test). The significant value of slope observed for GR ($p<0.05$; ANCOVA) means that, for a same FLU concentration, the GR response is different according to the

dietary conditioning. This is of great significance because diet is here modulating the biomarker response to PAH. Regarding GST, although it was significantly affected by FLU exposure in ANCOVA analysis, it was not significant with Student's-t test (p=0.09).

Table 20. ANCOVA analyses of mussel biochemical biomarkers in relation with mussel FLU concentration, after 7 days of exposure to FLU.

Biomarker	Factor	DL	F	p-value
CAT				
	FLU concentration	1	6.06	**0.02**
	Intercept	1	12.38	**0.00**
	Slopes	1	0.17	0.68
SOD				
	FLU concentration	1	9.46	**0.01**
	Intercept	1	6.29	**0.02**
	Slopes	1	1.53	0.23
GR				
	FLU concentration	1	17.11	**0.00**
	Intercept	1	10.38	**0.00**
	Slopes	1	6.92	**0.02**
GST				
	FLU concentration	1	7.77	**0.01**
	Intercept	1	1.31	0.27
	Slopes	1	0.15	0.70
LPO				
	FLU concentration	1	0.76	0.39
	Intercept	1	1.51	0.23
	Slopes	1	0.02	0.90

SOD: Superoxide-dismutase, CAT: Catalase, GR: Glutathione Reductase, GST: Glutathione-S-transferase and LPO: Lipid Peroxidation. Significant differences of ANCOVA at a significant level of $p<0.05$ are marked in bold.

5. Discussion

5.1 Effect of diet quality on the biology of mussels

The main objective of this study was to assess the effect of primary production quality on some biological responses used as indicators of pollution. For that purpose, mussels were fed with the same energetic ratio of two algae, a diatom and a dinoflagellate, which differed in important biological and biochemical characteristics such as their biological structure and biochemical composition (Table 16). Despite the fact that algae were quite different, mussels from the two dietary conditions did not differ in their biochemical composition (total lipids, carbohydrates and proteins) indicating that mussel energetic needs were met regardless of the diet quality supplied. Specifically, fatty acid analyses revealed that polyunsaturated fatty acids (PUFA) were the largest group of FAs found in mussels (45.5 and 53% for *C*-mussels and *H*-mussels, respectively) including the eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) as major components, as reported by Freites *et al*. (2002). The (n-3) PUFAs, EPA and DHA have been mentioned to be essential for optimal development and growth of bivalves (Knauer and Southgate, 1999). In our experiment, mussels fed with *C. neogracile* showed higher values of 14:0, 16PUFA and EPA in the FA profile, in both studied fractions (NL and PL), clearly reflecting the FA profile of the diet. On the other hand, mussels fed with *H. triquetra* increased their FA content of 16:0, 18:0, 18PUFA and 22PUFA, being the ratio 22:6n-3/20:5n-3 significantly higher in these mussels. These results, are in agreement with other authors which reported higher values of FA 16:0, 18MUFA and DHA, together with the ratio 22:6n-3/20:5n-3>1, as major dinoflagellate markers (Budge *et al*., 2001; Dalsgaard *et al*., 2003). The only fatty acid that appeared in high concentrations in *H. triquetra* and was not detected in mussels´ lipid profile was 18:5n-3, possibly because mussels were not able to assimilate it or because of it was elongated into 20:5n-3. Some authors have suggested that this fatty acid, in other algae species such as *Gymnodinium spp.*, has a toxic effect in animals (Sola *et al*., 1999). In conclusion, our data corroborated findings obtained in several studies that observed how the phytoplankton communities in the field (Budge *et al*., 2001; Irisarri *et al*., 2014; Parrish *et al*., 2005) or the microalgae diets in laboratory experiments (Albentosa *et al*. 1994, Delaporte *et al*., 2005) are reflected in changes of the fatty acid proportions in primary consumers.

Table 21. Fatty acid composition of mussels under two dietary conditioning: diatoms (*C. neogracile*) and dinoflagellates (*H. triquetra*) after 7 days of exposure to fluoranthene (FLU). Fatty acid analyses were performed in the neutral lipid (NL) and Polar lipid (PL) fractions of mussel's digestive glands and expressed as the molar percentages of total fatty acid content of each fraction.

	Digestive gland of mussel fed *C. neogracile*				Digestive gland of mussel fed *H. triquetra*			
	CONTROL		FLU		CONTROL		FLU	
Fraction	NL	PL	NL	PL	NL	PL	NL	PL
TMTD	1.7	1.9	1.0	2.6	2.5	2.0	1.4	1.3
BRANCHED	0.8	1.7	0.8	1.34	0.8	1.5	1.0	1.5
14:0	10.2	2.4	10.1	2.6	3.9	1.2	4.0	1.1
16:0	14.1	10.1	15.7	12.2	15.5	11.8	25.3	10.2
18:0	2.1	4.4	2.7	5.1	3.0	4.2	13.5	4.5
OTH. SFA	1.1	1.4	1.1	1.0	1.1	1.3	1.3	1.4
16:1n-9	0.2	0.5	0.2	0.4	1.3	0.7	1.0	0.5
16:1n-7	12.6	2.9	12.9	3.1	7.2	1.9	5.6	1.9
18:1n-9	0.9	0.7		0.7	1.6	0.8	1.8	1.0
18:1n-7	3.1	2.2	3.1	2.4	3.0	2.0	2.1	1.5
20:1n-11	0.8	1.5	0.8	1.1	1.4	1.5	0.9	1.7
20:1n-9	0.7	2.0	0.8	1.7	1.1	2.3	1.0	2.5
20:1n-7	1.0	1.1	1.1	1.1	1.1	0.9	0.9	0.9
OTH. MUFA	2.3	1.2	2.0	0.9	1.0	1.4	0.9	1.4
16:2n-7	3.8	0.7	3.1	0.6	0.2	0.0	0.1	0.0
16:2n-4	1.4	0.2	1.3	0.2	0.6	0.1	0.5	0.1
16:3n-4	4.0	0.8	3.4	0.6	0.4	0.5	0.3	0.6
18:2n-6	0.6	0.5	0.8	0.5	1.9	1.0	1.4	0.9
18:3n-3	0.5	0.1	0.7	0.3	2.2	0.8	1.7	0.3
18:4n-3	1.1	0.4	1.3	0.5	9.6	3.3	6.2	2.3
18:5n-3	0.1	0.1	0.2	0.1	0.5	0.2	0.3	0.3
20:4n-6	1.4	5.6	1.3	4.6	1.6	4.9	1.2	6.7
20:5n-3	19.0	18.0	18.6	18.7	12.7	11.3	9.7	11.1
22:2n-6	0.0	0.4	0.1	0.3	0.0	0.5	0.1	0.3
22:5n-3	0.6	1.4	0.6	1.3	0.8	1.2	0.7	1.5
22:6n-3	6.0	9.2	6.5	10.7	15.8	15.7	10.0	14.7
OTH. PUFA	5.9	3.5	5.3	3.4	4.8	3.4	3.9	3.5
NMI	2.5	10.2	2.2	7.8	3.4	9.4	2.4	10.3
DMA	0.6	13.5	0.4	12.7	0.4	12.8	0.5	14.6
UNKNOWN	0.9	1.4	0.9	1.4	0.5	1.2	0.3	1.1
SFA	27.5	18.3	29.6	20.9	23.5	18.6	44.1	17.2
MUFA	21.7	12.1	21.9	11.4	17.7	11.6	14.2	11.4
PUFA	46.8	51.1	45.4	49.6	54.5	52.3	38.5	52.8
TOTAL	100	100	100	100	100	100	100	100
PUI	1.9	1.9	1.9	2.0	2.4	2.1	1.7	2.0
PI	9.7	11.3	9.7	12.1	13.8	12.8	9.4	12.4

TMTD: trimethyltridecanoic fatty acids, SFA: saturated fatty acid, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, NMI: Non-methylene-interrupted fatty acids, DMA: dimethyl acetal fatty acids, PUI: Unsaturation index and PI: Peroxidation index.

5.2 Biomarkers

5.2.1 Bioaccumulation

Bioaccumulation results evidenced high accumulation of FLU by mussels regardless the dietary condition. In this present study, both algae were incubated with FLU for 45 min in the dark before being supplied to experimental tanks with a peristaltic pump. Considering that both mussel conditions were exposed to the same nominal FLU concentration, 30µg L^{-1}, the different bioaccumulation observed was an unexpected result. Different hypothesis could explain this fact:

First, existing differences in algae biochemical composition could partially explain the differences in mussel bioaccumulation. PAHs are lipophilic compounds, which means that this kind of compounds evidence high affinity to biological compartments with high lipid content. On the basis of total lipid data obtained from Table 16, higher values of total lipids and fatty acid were observed in *C. neogracile* per cell volume unit. As consequence, the final lipid content (mg ind^{-1}) supplied in *C. neogracile* dietary conditioning was 2-fold higher than in *H. triquetra* (see Table 16 for more details). This higher lipid content may promote a better adsorption of FLU by diatoms and therefore, be the cause of the higher FLU accumulation observed in C-mussels.

Another possible hypothesis is related to the feeding behavior of mussels. It is well known that mussel feeding behavior depends on various factors such as phytoplankton species composition and concentrations, cell size, shape and other structural features (Bayne, 1993; Navarro and Iglesias, 1993). As all food was cleared from the aquaria in the dietary conditioning, ingestion is assumed to be similar in both diets and cannot be claimed to explain FLU accumulation differences. However, there are some factors that may influence the absorption of algae ingested by mussels. For example, the small size of *C. neogracile* may facilitate its absorption and thus could explain the different accumulation of FLU observed in mussels. In this experiment, faeces were not collected, therefore, it is not possible to demonstrate this hypothesis, but, it was supported by previous studies carried out with *Mytilus edulis* (Rouillon and Navarro., 2003), in which mussels fed with a diatom and a naked flagellate, evidenced different absorption efficiencies. These authors explained that, even though all mussels ingested similar proportion of both microalgae, the ratio of algae changed dramatically in the stomach contents. In general, it seems that mussels have a preferential incorporation of diatoms possibly through a more efficient process of intracellular digestion. Since diatoms are characterized by a silicate frustule protection, it appears that they are more accessible to mechanical treatment by the crystalline style than flagellates, which are enclosed by a more fragile cell wall (Rouillon and Navarro, 2003). This study is also in agreement with previous results suggesting that diatoms' inflexible spicules seem to have a beneficial grinding effect of mineral silt particles in the gut of *Mytilus* as regards the digestibility of phytoplankton (Navarro *et al*., 1996). The better

efficiency assimilation of diatoms may favor toxicant accumulation and could be responsible of the different values of bioaccumulation observed.

5.2.2 Immune parameters

Effective immune responses and homeostasis are important in the maintenance of the organism health in bivalves, and rely on efficient functioning of the hemocytes (Pipe and Coles., 1995; Donaghy *et al* 2009). Hemocytes participate in a variety of functions including innate immunity, gas exchange, osmoregulation, nutrient digestion and distribution, waste excretion and wound repair (Cheng, 1981). In this context, some differences in immune responses were observed between dietary conditioning. The percentage of dead hemocytes were higher in *H*-mussels compare with *C*-mussels after the exposure period, but the differences observed, despite being statistically significant, may not be biologically relevant (2.0% and 2.8% in *C*-mussels and *H*-mussels respectively). Functional hemocyte parameters (phagocytosis and ROS production) were not or only partly affected by dietary conditioning.

On the other hand, some relevant effects of FLU were observed in immune parameters in response to FLU exposure. Percentage of dead hemocytes increased in exposed mussels after the exposure period. Similarly, Giannapas *et al*. (2012) evidenced a significant increase of percentage of dead hemocytes when mussels were exposed to phenanthrene (PH) and anthracene (AN) (at 0.1 mg L^{-1}) or their mixture (0.2 mg L^{-1}) for one week. Additionally, they observed a negative correlation between percentage increase of dead hemocytes and superoxide production. Authors interpreted it as a sign of alteration of the immune efficiency of mussels. The values reported by Giannapas *et al*., (2012), around 80-60 % higher percentage of dead hemocyte in exposed as compared to control treatments was much more drastic than those observed in the present study (2-3 %). In addition, significantly higher values of phagocytic capacity and hemocyte ROS production were observed upon FLU exposure in mussels fed *C. neogracile*. It has been reported that phagocytosis is more likely to undergo modulation on exposure to lipophilic organic contaminants, affecting cell membranes, rather than on exposure to inorganic metals (McCormick-Ray, 1987). The different FLU accumulation recorded in mussel tissues (2-fold higher in *C*-mussels compared to *H*-mussels)can be the responsible for the higher immune response activation observed. Phagocytosis and ROS production are tightly related. During phagocytosis, there is an increase in oxygen consumption and an increase in production of ROS, such as superoxide anion (O^{-2}) (Cazenave *et al*., 2006). Thus, the higher phagocytic activity could lead to higher ROS production in association with FLU metabolism, which involves ROS production (Stegeman and Lech., 1991). Despite contradictory results have been observed for immune responses of bivalves in presence of pollution, our results are in agreement with previous bivalve studies carried out in presence of PAHs (Renault, 2015). In general, a decrease of phagocytosis and hemocyte ROS production is observed when PAHs are present. Specifically, field studies carried out with mussels in presence of PAHs, showed a negative correlation between hemocyte phagocytosis and pollution (Sami *et al*., 1992). Conversely, different patterns in immune responses

have been observed in laboratory studies depending on the toxicant, the concentration and duration of exposure tested. For example, in a previous work by Grundy *et al.* (1996), phagocytic activity was reduced by a cocktail of PAHs (anthracene, fluoranthene and phenanthrene) at 750 µg L^{-1} for a period of 2 and 4 weeks. In other studies (Coles *et al*., 1994), exposure to 200 and 400µg L^{-1} of the PAH fluoranthene increased superoxide release by mussel hemocytes (Coles *et al*., 1994). Laboratory experiments have also demonstrated that phagocytic levels in mollusks can be enhanced following short-term, low-level of contaminant exposure (as in our study), but tend to decline with higher concentration and longer time exposure (Pipe *et al*., 1995).

5.2.3 Biochemical biomarkers

Contrary to immune parameters, important differences in biochemical biomarkers responses were observed depending on dietary conditioning. Higher values of CAT, SOD and GR were found in *C*-mussels after exposure period. The differences between algae size, structure and lipid composition could be the responsible for different physiological and biochemical processes which could affect the antioxidant responses observed upon dietary modification. As commented before (section 4.1), mussels were fed with the same ratio but differences in algae biometrics and biochemical composition ended in higher total lipid ingestion, and also PUFAs content, supplied to *C. neogracile* dietary conditioning. It has been described that an enrichment of lipids, specifically PUFAs, elicits an increase of ROS production and lipid peroxidation in vitro (Mazière *et al*., 1999). From this perspective, previous studies reported higher antioxidant activities (SOD and CAT) in fish liver fed with high lipid levels (Rueda-Jasso *et al*., 2004). Higher content of lipids, which are commonly reflected in higher PUFAs content, are extremely sensitive to oxidation. Their sensitivity increased exponentially as a function of the number of double bonds per fatty acid molecule, and therefore, PUFAs are much more susceptible to be attacked by radicals that are SFA or MUFA (Naudi *et al*., 2013). In this sense, many studies have shown that high dietary-PUFA levels increase lipid peroxidation in fish tissues (Olsen and Henderson, 1997; Zeng *et al*., 2015). Membrane phospholipids are main susceptible site to oxidative damage because ROS are more soluble in the fluid lipid bilayer than in the aqueous solution (Moller at al., 2005; Gamliel *et al*., 2008). The susceptibility of membranes to oxidative damage depends on the degree of polyunsaturation level of FA that can be measured as peroxidation index (Hulbert *et al*., 2007). In this study, a higher value of peroxidation index was observed in *H. triquetra* (20.6 %) compared to *C. neogracile* dietary conditioning (8.2 %, Table 17). Although a strong difference was observed in peroxidation indices between diets, similar values were observed in mussel peroxidation indices (Table 21). The most relevant information provided by this index was that peroxidation indices increased in *C*-mussels whereas it decreased in *H*-mussels in comparison with its diet peroxidation indices. The metabolic pathways that mussels develop to met their energetic requirements drawing from extremely different algae, may promote the differences found in biochemical responses observed after exposure and depuration times.

Regarding the effect of toxicant, significant induction of CAT, SOD and GR were evidenced upon exposure to FLU for 7 days in *C*-mussels. Mussels have relative poor ability to metabolize contaminant such PAHs and therefore, accumulates them in tissues to levels greatly exceeding those found in ambient sea water (Johnson, 1980, Widdows and Donkin, 1992). Once PAHs have been taken up by an organism, they may be subjected to biotransformation reactions. In bivalve mollusks, PAH metabolism largely occurs through radical oxidation involving reactive oxygen species (ROS) (Stegeman and Lech., 1991), which can be generated at various stages along the metabolic pathway (Livingstone., 1991). Specifically, some PAHs such as FLU, in addition to binding with the biomolecules, can induce ROS production which results in oxidative damage (Al-Subiai *et al*., 2012; Coles *et al*., 1994; Lowe *et al*., 1995). SOD catalyzes the transformation of superoxide radicals to H_2O_2, and is the first enzyme to deal with oxyradicals (Pamplona and Constantini, 2011). After the conversion produced by SOD, the antioxidant enzyme CAT decomposes H_2O_2 at high rates, but presents low affinity to peroxide and is mainly used during peaks of H_2O_2 production or accumulation (Pamplona and Constantini, 2011). In this context, GR does not directly remove radicals from the cell, but it is considered as essential antioxidant enzyme since it reduces oxidized glutathione (GSSG) and maintains the GSSG/GSH balance, which is basic for cellular homeostasis and the operation of other enzymes (Regoli and Giuliani., 2014). During the biotransformation of $ROOH^-$, GSSG is formed and this provides a substrate for GR. In the present experiment, GR activity was positively correlated with FLU exposure indicating that GSH recycling is taking place probably to cope with the detoxification requirements imposed by FLU exposure (Rodriges *et al*., 2013). In this sense, similar results (increase of CAT activity) were observed in mussels exposed to a combination of PAHs (Eertman *et al*., 1995; Fernández *et al*., 2010; Porte *et al*., 1991; Sureda *et al*., 2011). Other authors have described higher levels of GR and SOD activities in mussel digestive gland, in the presence of benzo[a]pyrene (Cheung *et al*., 2001), or an increase of SOD in the presence of fluoranthene (Eertman *et al*., 1995). These results evidenced that mussels have the ability to eliminate H_2O_2 that is produced by SOD and other biotransformation processes.

It is important to highlight that those antioxidant enzymes were affected by both, diet (higher SOD, CAT and GR in *C*-mussels) and FLU exposure, suggesting that there is an interaction between these two factors. In previous studies carried out with mussels to assess the effect of different food ratios on biomarkers responses to pollution (González-Fernández *et al*., 2015b) the effect of mussel nutritive state was so high that it masked the effect of pollution in biomarker responses. In the present study, the hypothesis that food quality could act as a confounding factor in biomarker responses to pollution is supported by our results. Not only GR activity increased with FLU concentration in mussel tissues, but the rate of these increases (slopes) is also affected by diet conditioning. Indeed, ANCOVA analysis revealed that GR evidenced higher slope in *C*-mussels in correlation to accumulated FLU than in *H*-mussels. In other words, for the same FLU concentration in mussel tissues, GR response differed depending on mussel dietary conditioning. It evidenced, for the first time, that food

quality have an effect on antioxidant activity responses. This information is critical in field studies, mainly in large-scale monitoring programs, where food quality conditions varied greatly between sampling sites. Therefore, diet quality can influence biomarker responses to pollution acting as a confounding factor.

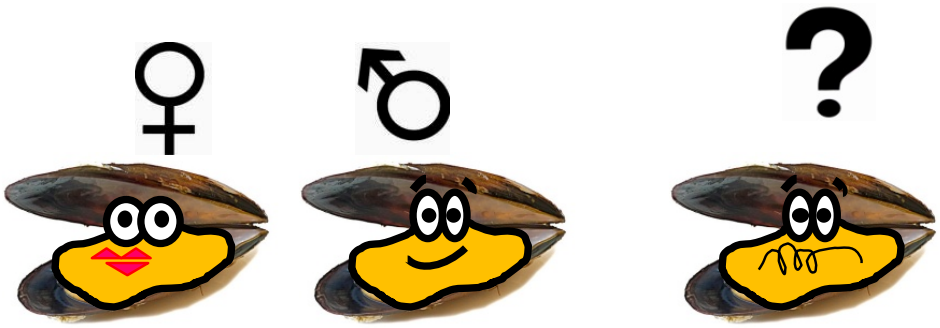
LPO were not affected by FLU exposure. The activation of a battery of antioxidant enzymes observed after exposure (SOD, CAT and GR) period may reflect the neutralisation of a substantial oxidative stress induced by the toxicant exposure as has been previously reported by several authors (Cheung *et al*., 2001; Fernández *et al*., 2010; Rodrigues *et al*., 2013).

6. Conclusion

The novelty of this study resides in the fact that, for the first time, food quality has been considered as a confounding factor in the evaluation of biomarker responses to pollution. After dietary conditioning, mussels resulted identical in CI and in their biochemical composition, only differing in FA composition. From an energetic point of view, it was assumed that mussels were similar. However, in spite of the fact that mussels were exposed to the same nominal toxicant concentration, accumulation of FLU, and consequently biomarker responses, were different. Algae characteristics (morphology, digestibility biochemical and fatty acid compositions) influence FLU absorption by mussels. Beyond difference of FLU accumulation, dietary conditioning could promote different biomarker responses as evidenced by GR activity. Food quality combined with other factors such as food availability (González-Fernández *et al*., 2015b) should be considered when understanding the biomarker responses to pollution. Fluctuations in "natural" environmental conditions can modify biological responses of organisms which are able to develop compensatory mechanisms making difficult the establishment of cause-effect relationships between pollution and biomarkers (Adams, 2005). This limitation is especially relevant in large-scale monitoring programs which cover a wide range of environmental scenarios (González-Fernández *et al*., 2015a). Because the main objective of monitoring programs is to establish the link between chemical pollution and harmful effects on marine organisms, trophic conditions should be integrated with chemical and biological data to better understand data variability at the ecosystem/geographical level.

CHAPTER 4

Condition III: Reproductive state



Carmen González Fernández, 2016

The research presented in this chapter forms part of the following publication:

González-Fernández, C., Albentosa, M., Campillo, J. A, Viñas, D., Franco, A., Bellas, J., 2015. Effect of mussel reproductive status on pollution biomarkers: Implications for large-scale monitoring programs. Aquat. Toxicol. (Submitted)

ABSTRACT

Biomarkers are useful tools to assess biological effects of pollutants and have been extensively used in monitoring programs to determine ecosystem health. In these programs, a wide range of environmental conditions are covered but data might be difficult to obtain and understand because of the affection of natural variables on biomarker responses. Previous studies carried out by our research group evidenced that different mussel reproductive states coexist in a monitoring survey. Thus, the main aim of this work was to identify the effect that mussel reproductive state has on biomarkers responses under standardized laboratory conditions (food and temperature). For that purpose, mussels sampled at two periods in the reproductive cycle (reproductive and resting stages) were conditioned to the same laboratory conditions to avoid the influence of external factors. After conditioning, mussels were exposed to fluoranthene (FLU) for three weeks. Mussel reproductive state was assessed by measuring gonadal development and reserves composition. Studied biomarkers covering a wide range of organism responses were included: bioaccumulation, physiological rates (clearance rate –CR-, absorption efficiency –AE-, respiration rate –RR- and their integration in the scope for growth-SFG-), antioxidant enzymatic activities (superoxide-dismutase -SOD-, catalase -CAT-, glutathione reductase –GR-, glutathione peroxidase -GPx-, glutathione-S-tranferase -GST-) and biochemical damage responses (lipid membranes peroxidation -LPO-).

The results obtained evidenced that the effect of mussel reproductive state was greater than toxicant in many of the biomarkers studied (RR, SOD, CAT and GPx), being their values higher at the reproductive stage. On another hand, the effect of toxicant was observed in SFG, CAT and GPx but this effect was only detected during the resting period. Moreover, there was a deterioration of mussel gonadal tissue with FLU exposure during the reproductive stage. FLU accumulation in mussel tissues was also dependent on the reproductive state, recording higher concentrations during resting periods. In conclusion, there was a strong effect of reproductive state on studied biomarkers which seems to mask the effect of FLU at reproductive stages. This paper demonstrates that the reproductive state has a strong influence on biomarker responses modulating pollution effects. The present study evidences, once more, the need to include the measurement of mussel biological parameters, as reproductive state, in marine pollution monitoring programs for a correct interpretation of biomarker data.

Key words: Biomarkers, mussels, reproductive state, fluoranthene, confounding factors, monitoring programs.

1. Hypothesis

Chapter 1 pointed out that mussel biomarker responses are dependent of mussel condition acting as a confounding factor hindering the use of biomarkers in monitoring programs. Mussel condition is dependent on both, nutrition and reproduction, which are closely associated. Moreover, results from Chapter 1 revealed that several mussel reproductive stages were coexisting in the same monitoring survey. The hypothesis of the present chapter is that mussel pollution biomarkers are affected by the mussel reproductive state.

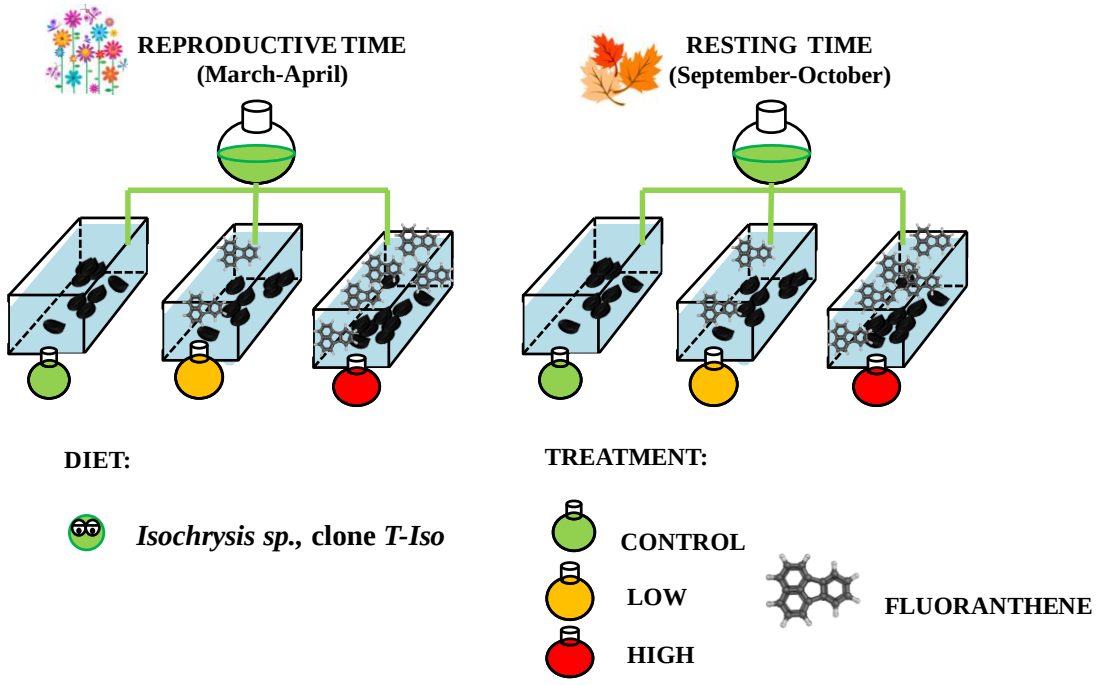
2. Objectives

The overall objective of this chapter was to assess, under laboratory conditions, the mussel biomarker responses to pollution along their reproductive cycle avoiding external environmental conditions of food and temperature.

The specific objectives of this chapter were to identify i) the effect of mussel reproductive state on a battery of biochemical and physiological biomarkers, ii) the combined effect of pollutant and mussel reproductive state on those biomarker responses, and iii) the implications of the obtained results in large-scale monitoring programs.

3. Summary of experimental design

Mussels were sampled at two periods in the reproductive cycle (reproductive and resting stages). They were conditioned to the same laboratory conditions to avoid the influence of external factors. After conditioning, mussels were exposed to two nominal concentrations of fluoranthene (FLU) for three weeks.



4. Results

4.1 Initial mussel condition

Mussel biological measurements before exposure, at the two reproductive stages (RS: February, reproductive stage and September, resting stage), are shown in Table 22. Biometric results evidenced that mussels were not significantly different, neither in length (44.9±1.9 and 45.8±2.0 mm, respectively; p=0.22) nor in total tissue dry weight (347.02±68.61 and 327.63±50.05 mg ind^{-1}, respectively; p=0.39). Thus, mussels were considered comparable in size and standardization of biometric data obtained in both experiments was not necessary. Tissue sizes was also similar between both seasons, being the only significant difference found in mantle and digestive gland. Reproductive mussels showed higher mantle and lower gland weights than resting mussels at the beginning of the experiment.

On the contrary, experimental mussels showed important differences at both reproductive stages in terms of gonadal development or biochemical composition of total tissues, in accordance with the experimental design. Regarding biochemical composition, main differences were related with CH with higher values at resting stage (62.53 mg ind^{-1}, being the double than reproductive stage, 31.30 mg ind^{-1}). Smaller, although significant differences were registered in PROT, with higher contents (229.37 mg ind^{-1}) at reproductive stage, this was around 30% higher than resting stage (165.73 mg ind^{-1}). LIPs were slightly higher during the resting period, although these differences were not significant (around 40 mg ind^{-1}).

Table 22. Biometric parameters, sexual development and biochemical composition (mean and standard deviations) of mussels (*Mytilus galloprovincialis*) used in the FLU exposure experiment at two phases of reproductive cycle: reproductive and resting stages. Tissues weights are expressed as mg of dry weight (dw) per individual. SMI: Sexual maturity index, RP: Reproductive potential for an individual standard of 1 g of dry weight. Biochemical composition is expressed as mg per individual and as mg per g of tissues organic matter (OM). Significant differences between and mussel reproductive stages at the beginning of the exposure experiment (noted as different letters) have been analyzed using the t-Student test at a significant level of $p<0.05$.

	Reproductive stage	Resting stage
Length (mm ind^{-1})	44.9±1.9 ^{a}	45.8±1.9 ^{a}
Tissue Weight (mg ind^{-1})		
Total	347.02±68.61^{a}	327.63±50.05^{a}
Gills	35.74± 4.22 ^{a}	38.91± 6.71^{a}
Mantle	62.42±20.92 ^{b}	35.25±15.20 ^{a}
Gland	55.23± 8.80 ^{a}	64.46±12.19 ^{b}
Remaining	188.16±49.47 ^{a}	183.09±29.44^{a}
Sexual development		
SMI	3.20±1.37 ^{b}	0.40±0.83 ^{a}
RP (mg gametes (g dw)$^{-1}$)	118.35±5.07 ^{b}	3.94±1.53 ^{a}
Biochemical composition (mg ind^{-1})		
Lipids	36.36±2.69 ^{a}	41.62±3.87 ^{a}
Carbohydrates	31.30±3.25 ^{a}	62.53±3.11 ^{b}
Proteins	229.37±3.26 ^{b}	165.73±5.66 ^{a}
CH/LIP	0.87±0.12 ^{a}	1.51±0.38 ^{b}
PROT/CH	7.38±0.76 ^{b}	2.66±1.78 ^{a}
Biochemical composition (% OM)		
Lipids	12.24±0.76 ^{a}	15.42±2.17 ^{b}
Carbohydrates	10.53±1.00 ^{a}	23.17±5.25 ^{b}
Proteins	77.23±0.74 ^{b}	61.42±4.79 ^{a}

The histological study showed substantial differences between both reproductive stages (Table 22). Mussels from reproductive stage displayed a high level of gonadal development compared with resting mussels, which were in an immature stage. Sex ratio (male:female:immature, Figure 34) in reproductive stages was 2.5:4:1, whereas at resting stage was 1:0:14. The percentage of undetermined individuals at initial time was 13% and 73% in reproductive and resting stages, respectively. The integration of gonadal size with the percentage of gonad tissues occupied by gametes, which we named mussel reproductive potential (RP), was completely different between both seasons, with a value of 118.36 mg gametes (g dw)$^{-1}$ at reproduction, whereas it was only 3.94 mg gametes (g dw)$^{-1}$ at resting stage.

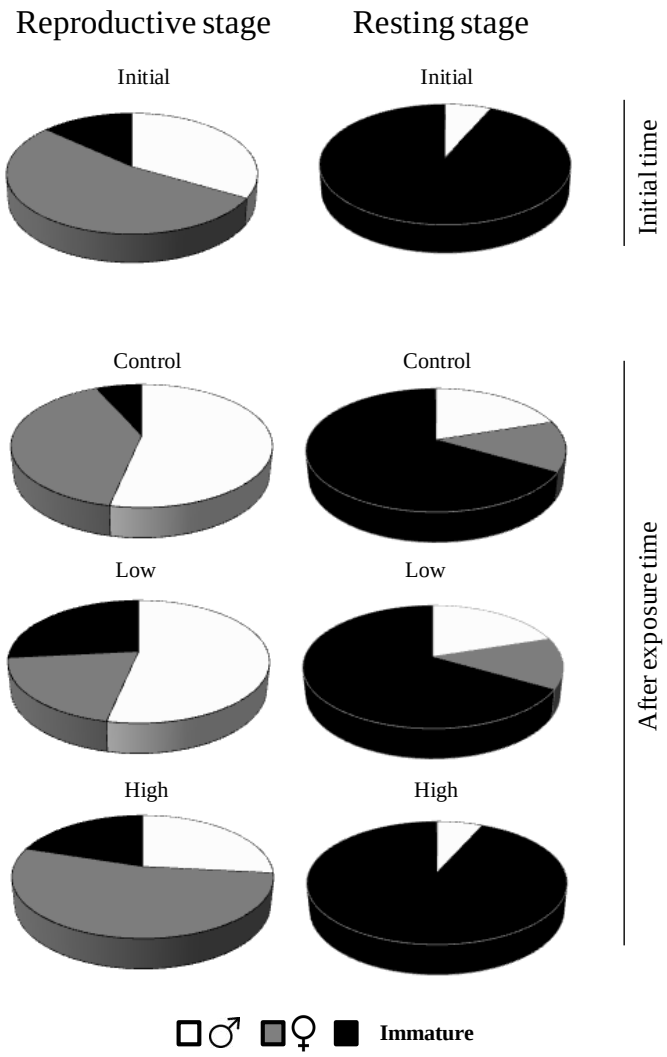


Figure 34. Graphic representation of sex ratio (Male, Female) and immature mussels (*Mytilus galloprovincialis*) percentages obtained in both reproductive stages sampled (Reproductive stage and Resting stage) at initial time and final times.

4.2 Final measurements

4.2.1 FLU effect on biometrics and biochemical components

Mean and standard deviations of biometrics after exposure conditions are shown in Table 22.

Table 23. Biometric parameters, sexual development and biochemical composition (mean and standard deviations) of mussels (*Mytilus galloprovincialis*) after the FLU exposure (Control, Low and High FLU concentration) at two phases of reproductive cycle: reproductive (RS-1) and resting stages (RS-2). Tissues weights are expressed as mg of dry weight (dw) per individual. SMI: Sexual Maturity Index and RP: Reproductive Potential expressed as mg of gametes per an individual standard of 1 g of dry weight. Biochemical composition is expressed as mg per individual. Significant differences between exposure treatments (lowercase letters) and mussel reproductive stages (capital letters) obtained from the multiple range test (LSD) on two-way ANOVA at a significant level of $p<0.05$.

	Tissue Weight					Sexual Development		Biochemical composition		
	Total	Gills	Mantle	Gland	Remaining	SMI	RP	Lipids	Carbohydrates	Proteins
			mg ind^{-1}				mg (g dw)$^{-1}$		mg ind^{-1}	
					Reproductive stage (RS-1)					
Control	376.4±58.8^{aA}	39.2±5.8^{aA}	81.3±33.1^{bA}	79.2±7.9^{aA}	172.3±24.0^{aA}	3.67±1.05^{aB}	166.5±4.8^{bB}	56.4±1.1^{abA}	37.7±4.6^{aA}	225.8±6.9^{bB}
Low	377.5±84.0^{aA}	40.1±6.9^{aA}	79.8±37.5^{bA}	78.4±18.4^{aA}	174.3±34.3^{aA}	2.93±1.83^{aB}	131.1±8.3^{abB}	60.7±2.8^{bA}	36.9±3.3^{aA}	221.6±5.0^{bA}
High	329.2±34.9^{aA}	39.2±5.4^{aA}	53.0±17.0^{aA}	73.5±9.3^{aA}	158.5±21.8^{aA}	3.07±1.62^{aB}	96.0±5.2^{aB}	49.8±5.8^{aA}	34.1±6.3^{aA}	191.7±2.5^{aA}
					Resting stage (RS-2)					
Control	433.9±68.6^{aB}	38.7±9.8^{aA}	76.7±15.4^{aA}	91.0±16.4^{aB}	221.5±37.0^{aB}	0.73±0.88^{aA}	12.2±2.2^{aA}	64.9±0.3^{aB}	100.1± 4.7^{aB}	211.8±1.7^{aA}
Low	437.9±68.8^{aB}	38.7±7.2^{aA}	75.9±26.4^{aA}	91.3±15.3^{aB}	225.2±37.8^{aB}	0.20±0.41^{aA}	0.0±0.0^{aA}	68.2±5.5^{abA}	102.9± 6.0^{aB}	213.7±4.3^{aA}
High	441.1±85.3^{aB}	40.7±8.4^{aA}	80.4±33.2^{aB}	94.4±13.6^{aB}	218.4±41.7^{aB}	0.33±0.41^{aA}	3.0±1.2^{aA}	76.6±4.6^{bB}	101.7±13.8^{aB}	211.6±8.7^{aB}

Despite the fact that mussels were identical at initial time (see section 3.1), and were kept under the same standardized conditions at both reproductive stages, mussels in resting stage significantly increased their weights, mainly due to the growth of glands, mantle and remaining tissues ($p<0.05$). As a consequence, resting mussels showed higher total dry weight (dw) than initial mussels for the three treatments ($p<0.001$), after 3 weeks. On the contrary, total dw of mussels at reproduction did not change significantly after experiment. Gill size which reflects mussel size, did not change during the experiment in none of the reproductive stages. Interestingly, for comparison purposes between reproductive stages, mantles were not significantly different than controls at the end of the experiment in both stages from a quantitative viewpoint, with weights around 80 mg ind^{-1}. For both seasons, the toxicant did not show any effect on the mussel biometry, excepting mantle size which decreased in reproductive mussels at high FLU concentration. This effect of FLU was not detected when mussels were at resting stage. F values from the two-way-ANOVA conducted with the biometric parameters were considerably higher for the reproductive state factor (F_{RS}) than for toxicant exposure (F_T): total ($F_{RS/}$ F_T=28.08/0.97), glands ($F_{RS/}$ F_T=26.59/0.05) and remaining tissues ($F_{RS/}$ F_T=56.82/0.92).

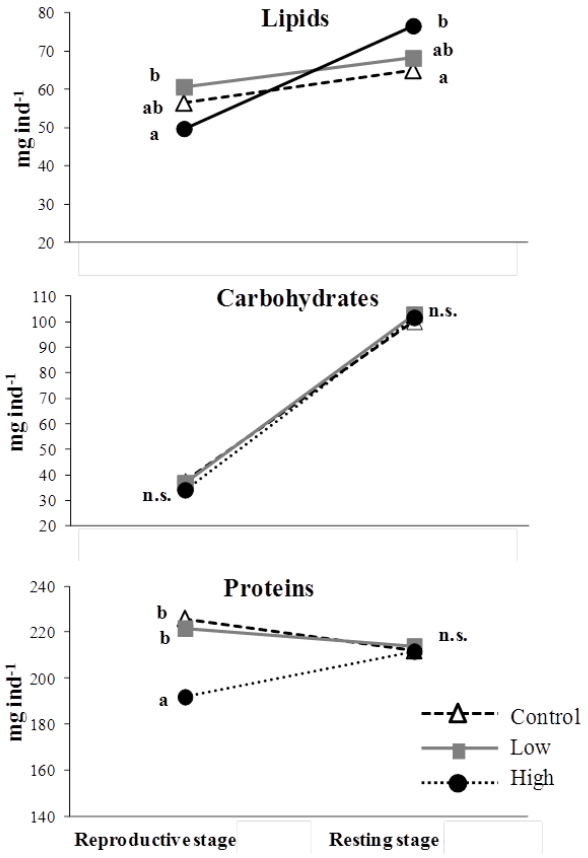


Figure 35. Gross biochemical composition (lipids, carbohydrates and proteins) of mussels (*Mytilus galloprovincialis*), expressed as mg ind^{-1} at two reproductive stages and exposed to two nominal concentrations of Fluoranthene (Low and High). Significant differences between exposure treatments obtained from the multiple range test (LSD) at a significant level of $p<0.05$ were noted with different letter.

Regarding biochemical components, statistical analysis also evidenced a strong effect of RS, as it was evidenced by F factor of the two-way-ANOVA, mainly for CH ($F_{RS/}$ F_T=358.46/0.11) but also for LIP ($F_{RS/}$ F_T= 58.49/1.41), which were present in a higher amount in resting mussels. Biochemical components are plotted in Figure 35. Whereas there was no effect of toxicant on CH, LIP were significantly affected by FLU exposure, although in a different way depending on season (F_{RS}*F_T=11.30, $p<0.05$). Whereas LIP decreased in mussels exposed at high FLU concentration during the reproductive stage, mussels in a resting stage increased their LIP (Figure 35). Regarding PROT, mussels showed higher levels while reproductive stage ($p<0.05$) affecting FLU exposure (F_T=18.88, $p<0.05$), decreasing at high FLU concentration. However, this effect was not observed when the mussels were at resting stage (F_{RS}*F_T=16.70, $p<0.05$).

4.2.2 FLU effect on mussel gonadal development

Two-way-ANOVA analyses carried out on histological results showed that both factors (RS and Toxicant) affected gonadal development, but showed again higher effect of RS: SMI ($F_{RS/}$ F_T=125.71/2.38), FVG ($F_{RS/}$ F_T=165.15/2.28) and RP ($F_{RS/}$ F_T=171.30/5.78), in such a way that the reproductive stage of mussels showed higher levels of gonadal maturity. Percentage of males, females and immature individuals at each RS and for the three treatments, are shown in Figure 34. Besides seasonal differences in sex ratio, with less than 7% of immature mussels at reproductive stages as opposed to almost 70% at resting stage, FLU exposure seems to increase the proportion of immature animals at both seasons. Moreover, FLU exposure reduced the mussel reproductive potential around 22% and 40% at low and high concentrations (Table 22). The effect of FLU on RP at high concentration seems to be more pronounced in females (38% reduction) than in males (25%) (data not shown).

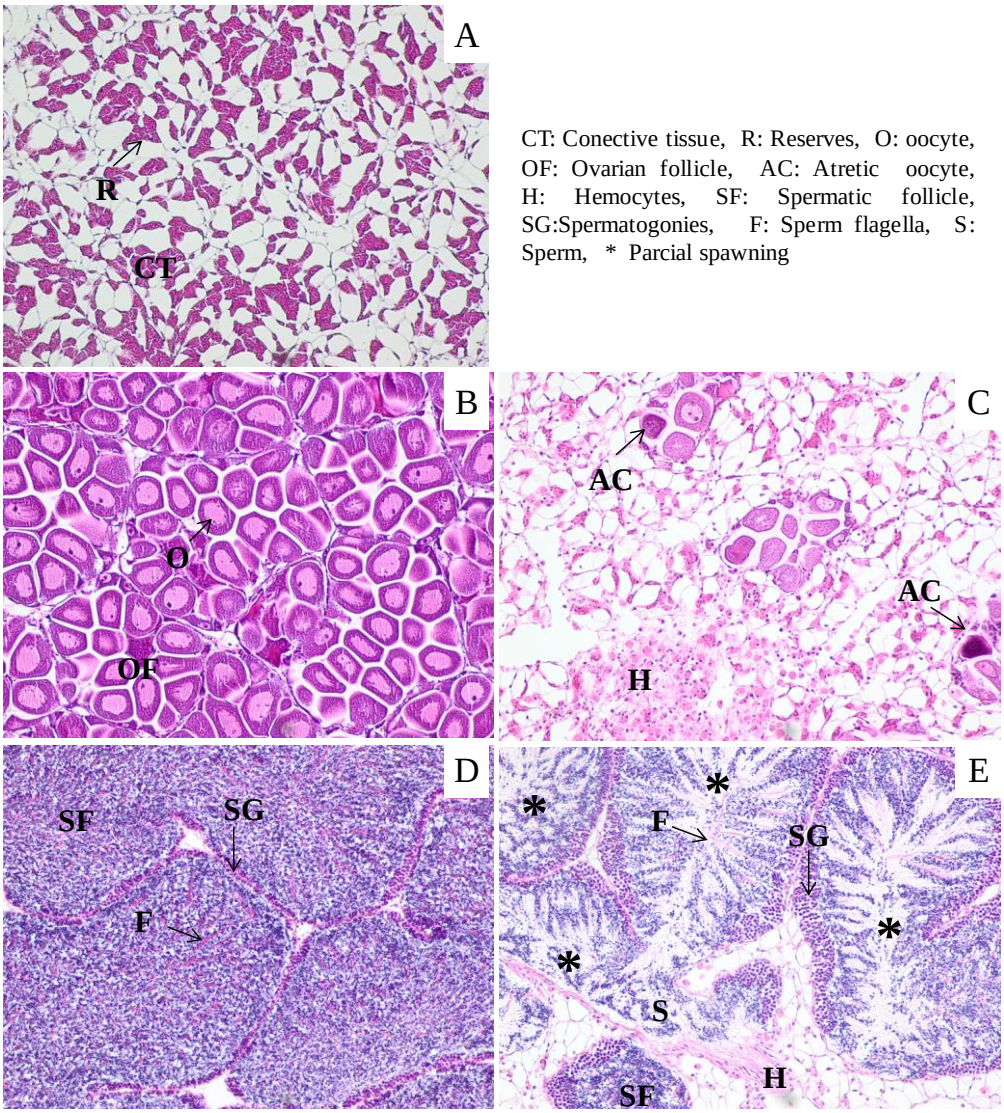


Figure 36. Micrographs showing gonad sections stained with hematoxylin/eosin from studied mussels. (A) Immature gonad section from control mussels at resting stage showing high storage tissue. (B) Female gonad section from control mussels at reproductive stage. (C) Female gonad section from High exposed mussels at reproductive stage showing severe haemocytic infiltration (arrows) of mature follicles. (D) Male gonad section from control mussels at reproductive stage. (E) Male gonad section from High exposed mussels showing severe haemocytic infiltration (arrows) and partial or total spawning (asterisks).

Histological micrographs (Figure 36) showed a negative effect of FLU on mature mussel gonads at the reproductive stage. At this stage, mussels showed mature gonads but there was a concentration-dependent damage in the gonads of exposed mussels. Mussels exposed to FLU showed symptoms of partial spawning, and those exposed to the highest FLU concentration, besides, showed gonad inflammation which was evident by the high frequency of hemocyte aggregates observed inside the gonadal connective tissue (Figure 36 C, E). As mentioned above, female mussels were more affected than males at high exposure. Histological samples of some individuals evidenced atretic oocytes (Figure 36 C).

4.3 FLU effect on biomarkers

4.3.1 Bioaccumulation in mussel tissues

Mussel FLU bioaccumulation is shown in Table 23. A two-way-ANOVA showed a dose-dependent increase of FLU accumulation in mussel tissues ($p<0.001$). Moreover, accumulation was different according to mussel reproductive state at high FLU concentration. Mussels exposed to high FLU concentration displayed the highest accumulation in their tissues at resting stage. Thus, FLU concentration was double (186.03 µg ind^{-1}) in mussels during the resting period than when mussels were mature (95.55 µg ind^{-1}). However, no significant differences in FLU accumulation were found at low exposure between RS (in both stages the FLU concentration was almost 5 µg ind^{-1}). Similar results were obtained regarding accumulation efficiencies at high FLU concentration, which were double at resting stage (31.89% of the FLU offered) in regards to the reproductive stage (15.36 %). It is worth highlighting that efficiencies were similar between low and high FLU concentrations during the reproductive stage whereas while resting, they were higher at high concentration.

Table 23. Fluoranthene accumulation (FLU) in mussel tissues (means and standard deviations) of mussels, *Mytilus galloprovincialis*, at two phases of reproductive cycle; reproductive and resting stages and exposed to two nominal concentrations of FLU (Low and High). Significant differences between exposure treatments (lowercase letters) and mussel reproductive stages (capital letters) obtained from the multiple range test (LSD) on two-way-ANOVA at a significant level of $p<0.05$.

Treatment	Concentration		Bioaccumulation Efficiency (%)
	Relative µg FLU (g dw) $^{-1}$	Absolute µg FLU ind^{-1}	
	Reproductive stage		
Control	0.03 ± 0.01^{aA}	0.01 ± 0.00^{aA}	---
Low	12.83 ± 1.45^{bA}	4.84 ± 0.55^{bA}	15.70 ± 1.78^{aA}
High	290.26 ± 95.34^{cA}	95.55 ± 31.39^{cA}	15.36 ± 5.04^{aA}
	Resting stage		
Control	0.05 ± 0.01^{aA}	0.02 ± 0.00^{aA}	---
Low	10.00 ± 1.30^{bA}	4.38 ± 0.57^{bA}	15.15 ± 1.97^{aA}
High	421.73 ± 39.85^{cB}	186.03 ± 17.58^{cB}	31.89 ± 3.01^{bB}

4.3.2 Biochemical biomarkers

Mean and standard deviations of mussel biochemical biomarkers are shown in Table 24 whereas the F statistics obtained through a multifactor ANOVA are plotted in Figure 37. This analysis evidenced that some biochemical biomarkers (SOD, CAT, and GPx) were affected by RS. However, neither RS nor FLU exposure affected GST, GR or LPO. In general, significantly higher values of SOD, CAT and GPx were observed when mussels were in a reproductive stage, more than two times the values observed in resting mussels. The effect of toxicant was significant in GPx and CAT, but it was dependent on the season as it was only observed in resting mussels. CAT activity increased with FLU exposure, although this effect was only evident at high FLU concentration. On the contrary, GPx followed a bell-shaped response to FLU, with an increase at low FLU concentration, and a decrease to control levels at high FLU concentrations.

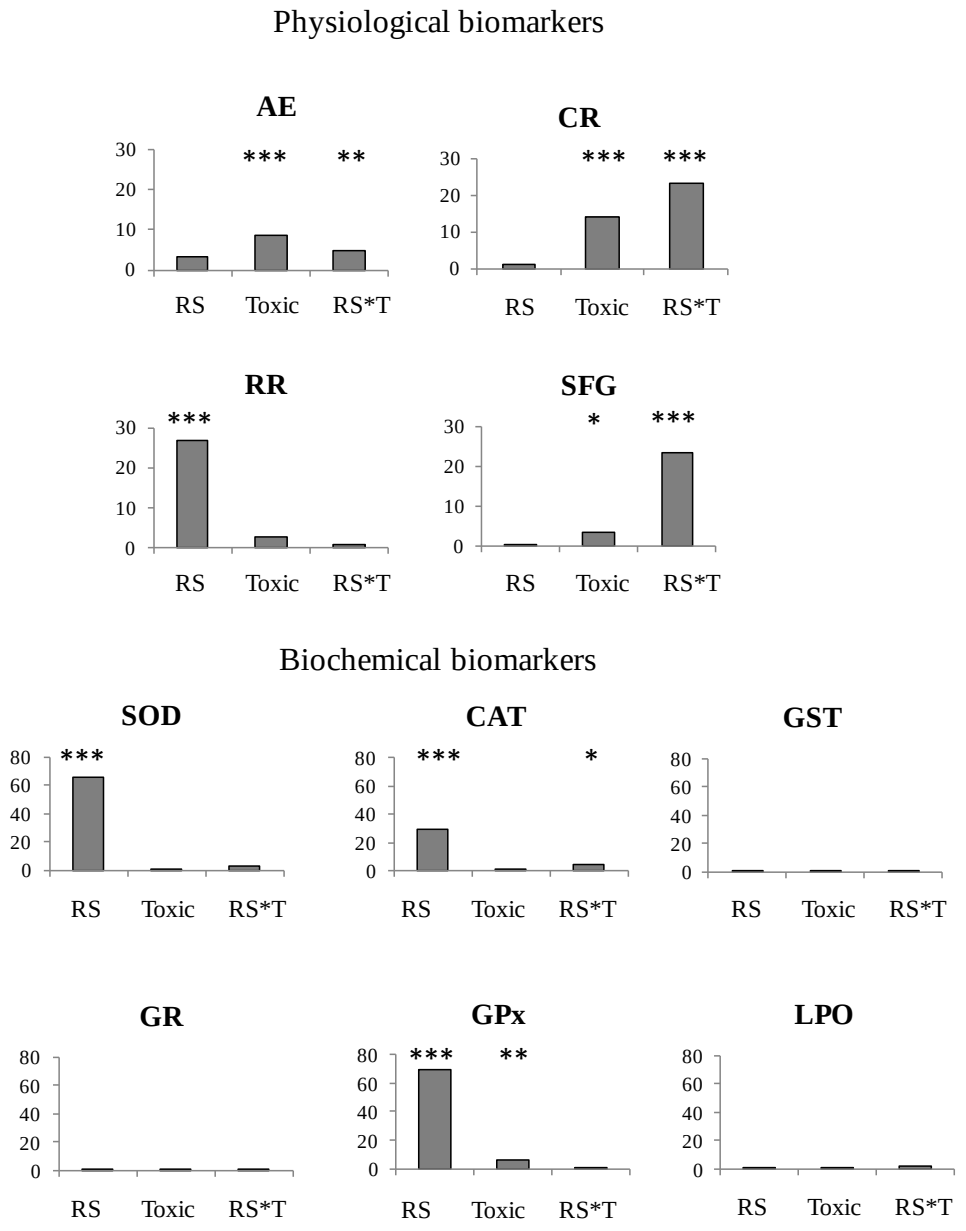


Figure 37. Results of the two-way-ANOVA on the physiological and antioxidant biomarkers responses. Bars showed the value of the F-test from the two-way-ANOVA being one factor the mussel reproductive stages (RS) obtained from the two different season sampling and the other factor the exposure to FLU at two doses (Toxicant). Interaction F values between both factors (RS*T) were also plotted. Significance of each F value was noted as *, ** or *** for a significance level of $p<0.05$, $p<0.01$ or $p<0.001$, respectively.

Table 24. Biochemical biomarkers (means and standard deviations) of mussels, *Mytilus galloprovincialis*, at different phases of reproductive cycle: reproductive and resting stages and exposed to two nominal concentrations of FLU (Low and High). SOD: Superoxide-dismutase, CAT: Catalase, GPx: Glutathione peroxidase, GR: Glutathione Reductase, GST: Glutathione-S-transferase and LPO: Lipid Peroxidation. Significant differences between exposure treatments (lowercase letters) and mussel reproductive stages (capital letters) obtained from the multiple range test (LSD) after one-way ANOVA and T-Student tests at a significant level of $p<0.05$.

Treatment	SOD	CAT	GPx	GR	GST	LPO
	U (mg prot)$^{-1}$	µmol min^{-1} (mg prot)$^{-1}$	nmol min^{-1} (mg prot)$^{-1}$			nmol MDA (mg prot)$^{-1}$
Reproductive stage						
Control	64.03±4.43^{aB}	81.83±24.09^{aB}	3.32±0.61^{aB}	35.66±5.46^{aA}	76.52±11.35^{aA}	4.06±1.18^{aA}
Low	70.50±7.73^{aB}	81.6±13.80^{aB}	4.63±0.94^{aB}	35.91±2.94^{aA}	78.93±18.84^{aA}	2.59±0.30^{aA}
High	70.20±5.90^{aB}	62.80±20.30^{aA}	4.08±0.82^{aB}	39.20±3.63^{aA}	80.36±9.55^{aA}	2.89±0.96^{aA}
Resting stage						
Control	49.31±10.35^{aA}	37.11±8.88^{abA}	1.62±0.36^{aA}	37.71±8.50^{aA}	84.10±11.66^{aA}	3.09±0.81^{aA}
Low	36.61±11.26^{aA}	30.79±6.67^{aA}	2.45±0.60^{bA}	35.88±2.89^{aA}	84.05±11.39^{aA}	3.68±1.42^{aA}
High	40.82±7.29^{aA}	52.98±16.80^{bA}	1.55±0.50^{aA}	44.75±13.68^{aA}	82.78±20.65^{aA}	2.94±0.78^{aA}

3.3.3 Physiological biomarkers

Mussel physiological biomarkers (mean and standard deviations) are shown in Table 25. On the basis of physiological parameters considered in this study (CR, AE, RR and SFG), only RR was significantly affected by RS in control mussels, which was significantly higher during the reproductive stage. CR, AE and, consequently SFG, were affected by FLU exposure, although the effect of the toxicant was dependent on the mussel reproductive state (results of the two-way-ANOVA are shown in Figure 37). There was a dose-dependent decrease in the CR with FLU, but this effect was only evident when the mussels were immature (resting stage). On the contrary, when mussels were mature, we only observed the decrease on the CR with low FLU concentration. Despite this effect, CR differences associated to FLU exposure were considerably less important than in resting stage. In fact, the light decrease of CR in low FLU concentration for reproductive stage mussels did not cause any effect on SFG. Despite AE was significantly affected by FLU at resting time, with low exposed mussels displaying the highest AE, no significant differences were observed between control and high concentration. Therefore, no conclusive remark can be made about the effect of FLU exposure on food absorption. No effect of toxicant was found in RR at any season. Finally, a severe effect of toxicant on SFG, which integrate all the physiological parameters, was evidenced at high concentrations (C=L>H), with a 6-fold reduction with respect to control values (12 for control and 2 J g^{-1}h^{-1} for high mussels).

Table 25. Physiological parameters (mean and standard deviations) of mussels (*M. galloprovincialis*) at two phases of reproductive cycle; reproductive and resting stages and exposed to two nominal concentrations of fluoranthene (Low and High). Physiological rates were measured under standardized laboratory conditions (15ºC, filtered seawater 1 µm, 0.8 mg L^{-1} of algal cells and 0.5 mg of inorganic matter L^{-1}). CR: clearance rate standardized to 1g gram of gill dry weight, IR: ingestion rate, AE: absorption efficiency, AR: Absorption rate, RR: respiration rate and, SFG: scope for growth. Significant differences between exposure treatments (lowercase letters) and mussel reproductive stages (capital letters) obtained from the multiple range test (LSD) on two-way-ANOVA at a significant level of $p<0.05$.

Treatment	CR	IR	AE	AR	RR	SFG
	L h^{-1}	J g^{-1} h^{-1}	%	J g^{-1} h^{-1}		
			Reproductive stage			
Control	13.52±1.82 ^{bA}	34.53±2.35 ^{bA}	58.00±5.16 ^{aA}	20.03±2.25 ^{abA}	9.74±1.60 ^{aB}	10.28±2.36 ^{aA}
Low	11.41±2.86 ^{aA}	29.55±7.75 ^{aA}	61.19±5.49 ^{aA}	18.11±5.05 ^{aA}	8.66±1.50 ^{aA}	9.45±5.91 ^{aA}
High	14.37±2.65 ^{bB}	39.71±3.99 ^{bB}	58.86±4.01 ^{aB}	23.42±3.26 ^{bB}	10.05±0.98 ^{aB}	13.37±2.55 ^{aB}
			Resting stage			
Control	16.67±2.74 ^{bA}	36.88±4.65 ^{cA}	53.70±7.34 ^{aA}	19.88±4.08 ^{bA}	7.90±1.24 ^{aA}	11.99±4.92 ^{bA}
Low	14.03±5.32 ^{bA}	30.20±9.55 ^{bA}	65.73±4.82 ^{bA}	20.03±6.48 ^{bA}	7.62±0.94 ^{aA}	12.40±6.99 ^{bA}
High	9.35±2.67 ^{aA}	21.58±6.57 ^{aA}	47.24±15.51 ^{aA}	10.13±4.40 ^{aA}	8.019±0.45 ^{aA}	2.11±4.48 ^{aA}

3.4 Integrated Biomarker Response (IBR)

The Integrated Biomarker Response (IBR) index provided a graphical description of the mussel state based on biomarker responses integration obtained from both reproductive stages and the three treatments considered in this study (Figure 38). Values of control mussels at the reproductive stage were considerable higher in comparison to control mussels at resting stage. Instead, values of IBR of low and high exposed mussels did not differ too much from control mussels in this RS. In contrast, a negative effect of toxicant was evident in biomarker responses of resting stage high exposed mussels, with an IBR value 6 points higher (IBR=8.64) than low (IBR=1.56) and control (IBR=1.31) mussels. Even so, control mussels at reproductive stage (IBR=7.11) displayed a similar IBR value than high exposed mussels at resting stage, highlighting the strong effect of reproductive state on antioxidant activities normally used as pollution biomarkers (Figure 38).

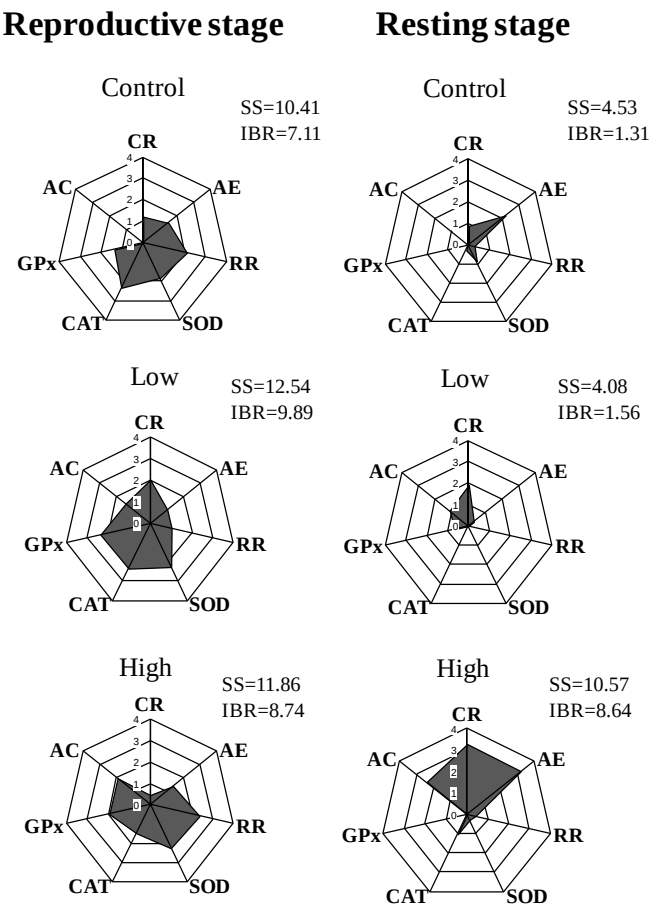


Figure 38. Radial representation of IBR calculation for selected biomarkers affected by reproductive stage or Fluoranthene. CR: Clearance rate, AE: Absorption efficiency, RR: Respiration rate, AC: accumulation, CAT: Catalase, SOD: Superoxide dismutase and GPx: Glutathione peroxidase.

4. Discussion

4.1. Mussel reproductive state

A general annual reproductive cycle has been described in mussels according to which gametogenesis takes place between late autumn and winter until the beginning of the spring, when the gonad is completely ripe and spawning occurs (Seed, 1976; Bayne, 1976, 1984; Lubet, 1959). In particular, in the Galician Atlantic coast, two reproductive cycles have been described, with an important spawning peak in spring and a second and less intense peak in autumn (September-October) (Cáceres-Martínez and Figueras, 1998; 2007). However, this general pattern differs markedly between nearby areas depending on food availability, as it was pointed out in the pollution monitoring program along the Spanish Atlantic and Cantabrian coast (González-Fernández *et al.*, 2015a). In open-sea areas, as those where the experimental mussels for this study were collected, food availability follows a more pronounced seasonal pattern than confined areas, therefore a seasonal reproductive cycle should be expected. By sampling the mussels at different seasons and acclimating them to standardized conditions, we obtained two groups of mussels from the same origin, with the same condition in terms

of meat weight, at two different seasons where different reserves content and gonadal development are expected. Thus, we obtained two well differentiated reproductive stages, mature reproductive mussels in February (reproductive stage) and a clear resting stage in September.

Regarding the biochemical reserves (storage reserves), significant differences in total CH values were observed between mussel stages, with doubled levels of CH at the resting stage. In bivalves, the reproductive cycle is closely related to the storage cycle (Gabbott, 1975). It has been widely described that energy components store mainly in the mantle during periods of sexual rest. Food abundance (stored in the summer) is used to fuel gametogenesis in late autumn and winter (Whittle and Gabbott., 1986). The percentage of CH obtained in this study which ranged from 10.53 to 26.74% of total organic matter, are in accordance with previous CH values found in mussels from this area during the whole (one-year) reproductive cycle, ranging from 5.47 to 38.48 % (unpublished data). CH contents at resting stage were similar to levels registered in autumn in areas from the Galician Atlantic coast (ca. 31%, González-Fernández *et al*., 2015a). Other authors have described similar patterns in mussel biochemical components, with higher CH contents in autumn and winter, when the metabolic demand is high due to gametogenesis, and a fall in glycogen reserves to a minimum value in mid-winter (January-March) (Bayne *et al*., 1976). Thus, CH and LIP usually follow an inverse relationship (Bayne *et al*., 1976), with higher CH/LIP ratio in autumn. In this study, mussels showed a CH/LIP higher relationship in the resting stage (1.47) compared to the reproductive stage (0.71). Biochemical composition is usually reported as relative percentages between the three main corporal components. The disadvantage of expressing the results as percentages is that changes in one component are reflected by the corresponding change in the other components (Gabbott, 1976). In the present study, biochemical data were also presented in absolute terms (mg ind^{-1}), which allows us to state that the main seasonal variation in biochemical components is related to CH. Higher CH/LIP ratios during resting stage are associated to the increased CH composition, whereas the decrease observed during reproduction is not due to an increase in LIP (of whole animal), but to the decrease of CH which have been consumed during gametogenesis.

In *Mytilus spp.*, the mantle plays an active role in gametogenesis, as it is the main site to storage glycogen reserves and the tissue where gonad develops. In addition, in the presence of food, protein and lipid reserves are also build up, mainly in non-mantle tissues (Gabbott and Bayne., 1973). Our results evidenced that during laboratory exposure (3 weeks), mussels at resting stage increased their total tissue dry weight, in particular, mantle, gland and rest (muscles and foot) tissues, which have been described as reserve storage tissues in mussels (Bayne *et al*., 1973). In *M. edulis*, the distribution of assimilated food is controlled by the digestive gland and, during summer and autumn, the transference of food to storage reserves takes place rapidly and is essentially completed within 7-10 days (Thompson and Bayne., 1972). This fact explains the increase of storage tissues observed in control mussels during exposure at resting stage.

On the contrary, mussels with very maturegonads (reproductive stage) did not increase (no significant differences) their corporal tissues, except for gland. During this season, mussels were in a high-energy-demand state due to gametogenesis requirements, as it was evidenced by their higher respiration rates. Under these physiological conditions, energy obtained from assimilated food should be used in maintenance, instead of growth. It is worth noting that no significant differences were found in mantle tissue dry weights between control mussels. This is an important result considering that strong differences in mussel reproductive state were observed. Higher values of SMI and % GVF were registered at the reproductive stage. The integration of these parameters, which were considered as mussel reproductive potential (RP), was also higher at the reproductive stage, supporting the high mature mussel state observed. This fact allowed us to perform more comparable analysis about the FLU effect on biomarkers at both seasons.

4.2 FLU damage on gonadal tissues

Hydrocarbons are known to have a wide variety of reproductive effects in bivalves, including atrophy of the germinal epithelium, reductions in the volume of gametes and storage cells, and increased levels of oocyte atresia (degeneration) (Lowe and Pipe, 1986a; Frouin *et al*., 2007; Jing-Jing *et al*., 2009). In this study, a negative effect of FLU was observed on mussel gonadal tissues on the reproductive stage. Mussels showed a clear reduction of the reproductive potential (RP) to the presence of FLU, which reached a decrease of 40% with respect to control, at high concentration. This effect was supported by the reduction of mantle tissues observed in these mussels. Partial spawning episodes might have occurred during the experimental period, mainly in the last days of exposure when the highest FLU accumulation was achieved. Decreases in mantle size might be related to the observed reduction of PROT, and to a lesser extent, LIP, in contrast to control mussels. Gamete emissions could be responsible for the above mentioned losses, since PROT and LIP are the main components of gametes (Gabbott, 1983).

There was a negative effect of FLU on mussel gonadal tissue during the reproductive stage. Mussels showed tissue deterioration, overall, at high FLU concentrations, with symptoms of inflammation and atrophy of the germinal epithelium. Similar effects on mussel reproductive tissue have been described by other authors (Aarab *et al*., 2008, 2011; Ortiz-Zarragoitia and Cajaraville, 2006), including a high frequency of hemocytic aggregates inside the gonad follicles and digestive glands, in mussels from a pyrogenic PAH contaminated site (Feist *et al*., 2006) or exposed to a mixture of oil PAHs and alkylphenols (Lowe and Pipe, 1986b, 1987). Furthermore, studies performed to assess the effect caused by an oil spill on mussel reproduction, as the one carried out by Ortiz-Zarragoitia *et al* (2011) after the *Prestige* spill in 2002, showed gonadosomatic damage in exposed mussels, with a high prevalence of oocyte atresia together with a high hemocytic infiltration on gonad follicles, especially for females. In fact, this is not an isolated result, because prevalence of female histological damage was evident after the *Prestige* oil spill in 2003, 2004, 2005 and 2006, being

considerably higher during the first year (Ortiz-Zarragoitia *et al*., 2011). In the same way, we observed a more pronounced reproductive damage in females than in males, with a 38% reduction of gonadal tissues compared to controls, in females exposed to high FLU concentrations, while a reduction of 25% was observed in males. These results are in agreement with previous studies (Baussant *et al*., 2011) chronically exposing mussels to dispersed oil. It seems that females are more susceptible to damage caused by organic pollution, probably due to the higher lipid content of their reproductive tissues (Bayne *et al*., 1975). Besides, although the effect of FLU was more evident in females, both sexes showed symptoms of partial emptying of the gonad at the reproductive stage.

Mussel mature follicles exposed to low and high concentrations of FLU were partially empty, despite no spawning evidence in aquaria. Lowe and Pipe (1987) investigated the effect of a chronic diesel oil exposure in mussel nutrient storage, *M. edulis*. Results indicated that hydrocarbon exposure led to a reduction in the levels of storage reserves and an increase in gamete atresia and re-absorption but, as a result, the storage pool was partially replenished and animals were able to tolerate the hydrocarbon's action. The study proposes re-absorption as a resistance strategy for the nutrient storage matrix in mussels exposed to low levels of hydrocarbons, involving the reallocation of reserves previously directed towards gamete production. The re-absorption of degenerate or atretic gametes has been evidenced by other authors (Baussant *et al*., 2011) and it is considered as a common process in mussel gonad (Newell, 1989). However, we do not believe that re-absorption processes were taking place in exposed mussels, as we did not observe any increase in reserve components, such as CH. Since we did not observe either spawning events during exposure, it might be possible that PROT and LIP depletions under FLU conditions, contribute to the higher metabolic demands of reproductive mussels and to the higher demand of energy in exposed mussels for detoxification reactions. Anyhow, we cannot discard that negligible spawning events occurred during exposure, as the release of reproductive structures has been described in response to stressful conditions associated to pollution (Kluytmans *et al*., 1988; Cajaraville *et al*., 1991, 1992), which might be considered as a population survival strategy.

On the contrary, no effect of FLU on mantle tissue was observed in resting mussels. Since mussels at this reproductive stage did not present matured follicles, no gametes can be released under the presence of the chemical stress. At this stage, mussel mantle was completely full of storage tissue, mainly composed by CH.

4.3 FLU Bioaccumulation in mussel tissues

The bioaccumulation ability of contaminants in bivalves, such as PAHs, has commonly been reported as being greater than that of other comparable marine invertebrates, and for this (and other reasons) they have been used worldwide as sentinels in pollution monitoring programs (Livingstone, 1991). In this work, mussels exposed to 3 µg L^{-1} accumulated between 10-13 µg g^{-1} dw, whereas those exposed to 60

µg L^{-1} exhibited a FLU concentration between 290-422 µg g^{-1} dw. Moreover, FLU bioaccumulation efficiencies during the reproductive stage were similar to those obtained in a previous study carried out in our laboratories with the nutritional state as the experimental variable (González-Fernández *et al*., 2015b). However, efficiencies registered during the resting stage were double than those of the cited study. These differential FLU accumulation efficiencies would be expected, since experiments were not carried out at the same season (summer in the referred study and spring and autumn in the present one). In any case, FLU concentrations obtained in both laboratory studies might be comparable with those registered after a main oil spill. For example, as mentioned above, after the *Prestige* wreck close to the Atlantic Spanish coast, mussels tissues had PAHs concentrations around 8 µg g^{-1} dw (sum of parent 13 PAHs) (Soriano *et al*., 2006), or the *Sea Empress* oil spill, in Wales (UK), caused a PAHs accumulation (sum of 18 parent PAHs) in mussels exceeded 500 µg g^{-1} dw (Law and Kelly, 2004).

Furthermore, results from the present work showed different patterns of FLU bioaccumulation according to mussel reproductive state. Thus, mussels exposed to high FLU concentrations during resting time, showed higher FLU concentrations and accumulation efficiencies (almost the double of mussels at reproductive stage). Seasonal differences in FLU accumulation might be explained, in part, by mussel growth differences. Mussels ingested the same food ratio at both seasons, but they grew more at the resting stage. Thus, faecal and/or metabolic energy losses must be higher during reproduction. Had it been so, a higher proportion of microalgae polluted by FLU would have been removed through faeces, and/or higher metabolic demands would have occurred in mature mussels, increasing the combustion of polluted assimilated food.

An alternative/complementary hypothesis for the higher FLU accumulation on resting mussels would be related to the chemical properties of the pollutant used in the experiment. FLU is a highly hydrophobic compound which tends to bind to the nonpolar mussel components, as LIP, especially to neutral lipids (Jorgensen *et al*., 1997), which constitute most egg reserves in marine bivalves (Bayne *et al*., 1975). In this study, no significant differences were found in total LIP contents between mussel RS, probably because lipid analyses were performed in whole mussel tissues and not in the reproductive tissue (mantle). It has been described that lipid content is much higher in the eggs than in the body (Lotufo *et al*., 1998). Thus, taking into account that mussels at reproductive stage were completely full of gametes, it could be hypothesized that FLU content in gonads during reproduction would be higher than in mantle at resting stage. Then, the partial spawning episodes that have been hypothesized responsible for gonadal losses in reproductive mussels exposed to FLU, could be responsible for the lower accumulation in these mussels at the high concentration treatment. As commented above, mussels at this condition reduced their RP about 40% compared to control mussels, and showed gonadosomatic deterioration. Similar behavior has been observed in *M. edulis* exposed to cadmium, in which, the presence of cadmium has a stimulating effect on the spawning frequency (Kluytmans *et al*., 1988). Mussels exposed at low concentration did not show either gonadal losses compared to the control or a lower

FLU accumulation with respect to resting mussels, which strengthens the above mentioned hypothesis.

Differences in pollutant bioaccumulation along the year have been previously reported by other authors in mussels exposed to PCBs (Hummel *et al*., 1989, 1990) or to PAHs (Bourgeault and Gourlay-Francé, 2013). In these studies, concentrations of PAHs varied significantly between seasons, with minimum values obtained during gametogenesis and post-spawning and maximum values reached during the period of sexual rest and pre-spawn. This seasonal pattern has also been reported in other studies (Menone *et al*., 2000; Barni *et al*., 2014) which recorded temporal variations in the concentrations of organochlorine pesticides during the gonadal development of silverside fish *Odontesthes bonariensis*. Furthermore, these finding was supported by a contaminant dilution effect, linked to ovaries growth prior to spawning. Gundersen *et al*., (2000) described this process, in which the contaminant content of the gonads of fish became diluted as an egg mass developed and the size increased. This dilution effect cannot be applied to the results presented in this study, since mussels with higher FLU concentration (resting mussels) were the ones with higher growth.

4.4 Biochemical biomarkers

In seasonal variations of oxidative stress, biomarkers have often been related to changes in food availability, temperature and pollution, but not to the reproductive cycle *per se* (Palais *et al*., 2012, Hagger *et al*., 2010; Viarengo *et al*., 1991, Bocchetti and Regoli., 2006; Nahrgang 2013; Vidal-Liñán *et al*., 2010). These factors are bound up in mussels, since the spawning process seems to be highly dependent on environmental circumstances; the time and duration of the spawning season can be correlated with latitude (Lubet, 1959). As previously mentioned, mussels have an extended spawning season at low latitudes, with not well defined resting period due to stable temperatures. In these areas, spawning is influenced by chlorophyll peaks, which indicates a close relationship between food availability and the production of ripe gametes (Bayne, 1976; Lubet, 1959; Pieters *et al*., 1980). In this sense, studies carried out by our research group (Bellas *et al*., 2014; González-Fernández *et al*., 2015a) in monitoring programs (Dowd *et al*., 2013), pointed out that food availability plays an essential role on the enzymatic capacities for ATP generation and detoxification of the reactive oxygen species (ROS) in intertidal mussels, at the time scales covered. Moreover, these results support our previously referred study (González-Fernández *et al*., 2015b) designed to assess the effect of mussel nutritive state on biomarker responses. In that work, a clear effect of mussel nutritive state on biomarker responses was evidenced, and nutritive-stressed mussels showed the highest activities of antioxidant biomarkers (SOD, CAT and GPx). In the present study, it was essential to exclude the nutritive condition from the experimental design in order to ascertain the effect of reproductive success on biomarker responses. Therefore, the novelty of the present study lies in the complete isolation of reproductive condition from nutritive conditions by keeping mussels under the same standardized laboratory conditions for 1 month.

Results obtained in this study were comparable with the seasonal patterns of some antioxidant biomarkers (CAT, GST, GPx and total oxyradical scavenging capacity -TOSC-) reported for mussels *M. galloprovincialis* in the Adriatic sea (Bocchetti *et al*., 2008; Bocchetti and Regoli 2006; Borkovic *et al*.,2005), with higher activities in spring. Other studies have also reported higher antioxidant activities (CAT, GPx and GST) in blue mussels, with highest levels in March (Nahrgang *et al*., 2013; Schmidt *et al*., 2013), or lower activities GPx and GR in winter (Manduzio *et al*., 2004).

In the present study, mussels at reproductive stage evidenced higher values of antioxidant enzymes (SOD, CAT and GPx) that protect cells against the deleterious effect of oxyradical generation (Sheehan and Power, 1999). SOD eliminates superoxide radical by converting it to oxygen and H_2O_2, whereas CAT and GPx work coordinately to eliminate the H_2O_2 produced by SOD and other potential sources. CAT decomposes H_2O_2 at high rates, but presents low affinity for the peroxide and is mainly used during peaks of H_2O_2 production or accumulation. On the contrary, GPx decomposes H_2O_2 slowly but with higher affinity (Pamplona and Constantini, 2011). These antioxidant enzymatic activities are known to be under extensive seasonal control. Their activities in the digestive gland of mussels appear to be at their lowest values in winter, corresponding to increases in levels of lipid peroxidation products (Sheehan and Power., 1999). The increase of mussel metabolic rate attributed to gametogenesis was reported previously (Widdows and Bayne, 1971; Bayne, 1973). High investment in reproduction can generate a trade-off in the allocation of energy, leading to the detriment of important physiological functions. The energy demand is satisfied by an increase in the metabolic rate during the reproductive period (Angilleta and Sears, 2000). This results in an increase in intracellular respiration which can promote an elevation of ROS production (Béguel *et al*., 2013). On another hand, no significant effect of RS was observed on GST. This result was supported by previous studies in which no seasonal differences in GST were observed in mussel digestive gland (Power and Sheehan, 1996; Vidal-Liñán *et al*., 2010). No effect of RS was observed in LPO probably because the antioxidant enzymes activation provided lipid membrane protection.

Regarding the effect of the toxicant, an increase of ROS production in mollusks exposed to PAHs has been described (Solé *et al*., 1995; Porte el al., 2001a; Fernandez *et al*., 2010) and, therefore, in the antioxidant response of organisms. In this study, the effect of toxicant was only evident at the resting stage, where the higher accumulation of FLU was observed. An activation of the antioxidant enzymes CAT and GPx, which are strongly related to the H_2O_2 production (Regoli and Giuliani, 2014), was also registered in exposed mussels. Similar results (increases of CAT activity) were observed in mussels exposed to a combination of PAHs (Eertman *et al*., 1995; Fernández *et al*., 2010; Porte *et al*., 1991; Sureda *et al*., 2011). Other authors have described higher levels of GR, GPx and SOD activities in mussel digestive gland, in presence of benzo[a]pyrene (Cheung *et al*., 2001), or an increase of SOD in presence of fluoranthene (Eertman *et al*., 1995). These results evidenced that mussels have the ability to eliminate H_2O_2 produced by SOD and other biotransformation processes.

However, the increase of antioxidant activities under the presence of FLU was not evident in reproductive mussels, as they were already stimulated due to higher cell metabolism associated to gametogenesis.

4.5 Physiological biomarkers

Seasonal variations in physiological parameters of mussels have been described under natural field conditions. In particular, mussel CR showed a high degree of variability over tidal to seasonal time scales (Crandford *et al*., 2011). The most widely known process is that bivalves regulate their filtration rate and retention efficiency as a function of particle concentration (Hawkins *et al*., 2001). Phytoplankton availability in the Galician coast, where experimental mussels were collected, follows a seasonal pattern with higher chlorophyll concentration in the summer and fall (Babarro *et al*., 2000; Figueiras *et al*., 2002; Peteiro *et al*., 2011). As a consequence, feeding and absorption rates of mussels in this area generally show maximum values during the phytoplankton peak in spring while minimum values are registered in winter (Irisarri *et al*., 2014; Navarro *et al*., 1996). This fact points out the high flexibility inherent within bivalve species to alter feeding and absorption behavior over different temporal scales. It needs to be highlighted that the majority of these studies were carried out under natural field conditions. In the present work, on the contrary, mussels were acclimated to standard laboratory conditions of food and temperature (filtered seawater 0.5 μm, 15 °C, fed with a ration of 0.17% of microalgal organic matter (OM) per mussel live weight) for one week, and standard conditions were kept for three weeks, during the exposure period with a constant food supply. At the end of the experiment, despite mussels evidenced two different reproductive stages, they did not show significant differences in CR or AE.

Conversely, significant differences on mussel RR were observed between mussel reproductive stages. As feeding behavior, respiration is affected by environmental variables, and a regular seasonal pattern in the rate of oxygen consumption of some bivalves has been described (Bayne, 1976). In this study, higher mussels' RRs were registered during reproduction. Our results are in agreement with previous studies carried out with mussels from this area (Fernández *et al*., 2010; Irrisarri *et al*., 2014), which found maximum values of oxygen consumption in spring, while minimum values were found during autumn. This behavior was also reported in laboratory studies, in which oxygen consumption by *M. edulis* was measured throughout the year at a constant temperature (15 ºC) (Krüger, 1960, reviewed by Bayne *et al*., 1976). This study recorded a seasonal pattern of mussel oxygen consumption with high rates in spring, declining to minimum rates after spawning in summer. This author correlated the observed seasonal pattern with the cycles of gametogenesis and the storage and utilization of nutrient reserves. This relationship between gametogenesis, body reserves and routine rate of oxygen consumption has also been described by several authors (Bayne and Thompson, 1970; Widdows and Bayne, 1971; Bayne, 1973), and was supported by field studies (Rueda and Smaal, 2004). When mussels are at the "resting stage" and glycogen reserves are high, the high proportion of metabolically

inert material results in a low rate of oxygen consumption per unit of weight. During the active gametogenesis, the glycogen stored reserves are being utilized and the metabolic demand increases. In spring, a large mass of developing gametes continues to impose a high demand for oxygen which is only reduced after spawning.

Regarding the effect of the toxicant, although statistical analyses showed a significant effect on CR ($p<0.05$) at reproductive stage, with a reduction of CR on low exposed mussels, no biological effect was observed in this mussel condition during the three weeks of experiment. Conversely, a clear negative effect was registered at mussel resting stage. Mussel CR showed a dose-effect relationship, with a reduction of about 20% and more than 50% at low and high exposures, respectively. Most relevant physiological changes associated with exposure to organic pollutants, are those that may affect the organism´s growth and survival (McDowell *et al*., 1999). Alteration in growth potential may take place as a result of changes in feeding behavior, respiratory metabolism or digestive efficiencies. Some authors have reported that CR is the parameter most affected by pollution (Al-Subiai *et al*., 2012; Beiras *et al*., 2012; Toro *et al*., 2003). Moreover, similar results have been described by other authors in laboratory studies where mussels were exposed to a mixture of PAHs (Widdows *et al*., 1987; Widdows and Jonhson., 1988) or FLU alone (Eertman *et al*., 1995), evidencing a reduction of around 50% of mussels feeding rates. Donkin *et al*., (1990) suggested that decreases in feeding rates could be attributed to non-specific narcosis associated with the accumulation of low molecular weight hydrocarbons in the gills. The fact that only mussels at the resting stage showed an effect on CR could have several explanations. First, higher concentration of FLU was registered in mussels at this stage, being double than that registered in mussels at reproductive stage, which could be the responsible of the distinct physiological responses observed at these two reproductive stages. On the other hand, the greater energy consumption caused by the gametogenic effort could promote the need to maintain high feeding rates as a fundamental process to energy gain, even when FLU is present.

Furthermore, a significantly lower SFG of resting mussels exposed to high FLU concentrations was observed as a consequence of the decrease in CR. Alterations in bioenergetics and growth of bivalve mollusks related to oil-contaminated habitats have been described in field studies (Widdows *et al*., 1995; 2002; Fernández *et al*., 2010; Toro *et al*., 2003), and along contaminant gradients in laboratory studies (Widdows and Johnson, 1988; Widdows *et al*.,1995; Toro *et al*., 2003; Halldorsson *et al*., 2005). Moreover, a negative relationship has been established between PAHs accumulation in bivalve tissues and SFG, under laboratory conditions (Widdows *et al*., 1982; Donkin *et al* 1989; Kim *et al*., 2007). In the present study, only mussels exposed to the highest FLU concentration showed a decrease of SFG, being in accordance with the physiological behavior observed in previous studies. SFG data observed at low concentration was similar to that registered for controls, despite the lowered CR observed in these exposed mussels. The energy intake decrease was compensated by an increase in food absorption efficiency, in such a way that absorbed energy was similar

in both cases (control and low) and consequently, also the energy intended for growth (SFG).

6. Integrative assessment

In the present study, we attempted to identify the effect of mussel reproductive state on biomarker responses, avoiding the main exogenous factors governing gametogenesis: food availability and temperature. Our ultimate goal is to integrate this information for a better interpretation of biological data in coastal quality monitoring programs. An overall picture was obtained by the use of IBR, showing a masking effect of mussel reproductive state on biomarkers responses to FLU exposition. The increase of mussel metabolic rates associated to gametogenesis provokes an increase of ROS production, which induces a higher level of some antioxidant enzymes (SOD, CAT and GPx), clearly associated with increased oxygen consumption rates in these mussels. Some of these enzymes were also affected by FLU (CAT and GPx), and therefore, the effect of toxicant at the reproductive stage was masked by the interaction of both factors. Physiological biomarkers did not result significantly affected by FLU at mussel reproductive stage, although a dose-response was found at the resting stage, where a higher FLU accumulation was observed. Differential biomarker responses at the two reproductive stages may be explained in two different ways. 1. On the one hand, the high lipid content characteristic of mature gametes might facilitate a higher FLU accumulation in gonads which could be easily eliminated through partial spawning events. This decrease on FLU concentration caused by gamete emission might favor mussel fitness (no effect on SFG). Moreover, gamete emission could be a mechanism of population survival under adverse conditions maintained in the exposed aquaria. On the other hand, gametogenesis by itself increases cellular metabolism and therefore, ROS production, which is naturally compensated by the activation of antioxidant defenses and, consequently, the detoxification capacity of cells in the presence of toxicants is increased. Conversely, resting mussels use the mantle tissue to stora energetic compounds as carbohydrates, and accumulated FLU cannot be 'released'. In these mussels, the antioxidant machinery induced by reproduction is not present and fitness might be compromised as was evidenced by the reduction of SFG.

Previous studies carried out within our research group (González-Fernández *et al*., 2015b) evidenced that both, antioxidant activities and physiological biomarkers, are strongly affected by the mussel nutritive state, mainly the physiological biomarkers (CR), and in a lesser extent, antioxidant biomarkers (CAT and GPx). In the cited study, nutritive stressed mussels showed higher values of CR and antioxidant enzymes (SOD, CAT and GPx) than well fed mussels. In the present work, the same antioxidant activities were affected by mussel reproductive state. It seems that those antioxidant activities more related to ROS production are the most affected by mussel condition in both, nutritive and reproductive condition. Moreover, as it was observed in the present experiment, nutritive-stressed mussels accumulated less toxicant in their tissues than well-fed mussels. A mechanism of detoxification was suggested due to the activation of the autophagic system described by Moore *et al*. (2007; 2008). During gametogenesis,

gonad demands energy, therefore there is a mobilization of storage material from non-mantle tissues to the gonad. This mobilization implies, similarly to what observed during nutritive stress conditions, an activation of catabolic pathways in order to obtain the energy contained in storage tissues or directly from absorbed molecules through diet. In conclusion, mussels under particular biological situations (nutritive stress or reproduction) seem to be better equipped to combat toxic effect of pollutants. This fact may become a problem in field studies where both situations are present, as was evidenced in a large-scale monitoring survey (González-Fernández *et al*., 2015a).

Therefore, among other factors as nutritive state, the stage of the reproductive cycle needs to be taken into account in studies involving gland antioxidant defenses of mussels, especially considering the close functional relationship between digestive gland and gonad development (Sheehan and Power, 1999). The present study evidences, once more, the need to include the measurement of mussel biological parameters, as reproductive state, in marine pollution monitoring programs for a correct interpretation of biomarker data.

GENERAL DISCUSSION

Effect of confounding factors in biological responses of mussels, *Mytilus galloprovincialis*, to pollution: Implication in large-scale monitoring programs

Carmen González Fernández, 2016

GENERAL DISCUSSION

The **main objective of this thesis** was to determine the extent to which mussel endogenous factors may act as confounding factors of biomarker responses to pollution in large-scale monitoring surveys. The first approach of the thesis is described in Chapter 1, where a monitoring sampling survey was performed in the N-NW Spanish coast within the framework of the Spanish Marine Pollution Monitoring Program (SMP), including a comprehensive study of mussel biological parameters, in order to detect if **mussel biological differences** have an effect in the biomarker responses used as bioindicators of pollution. This monitoring survey includes two well-differentiated oceanographic regions, the Iberian Atlantic coast and the Cantabrian coast, which differ in their environmental conditions, mainly in food availability. The high complexity of marine ecosystems, where several variables may be acting at the same time, complicates the comprehension of biomarkers responses to pollution. Because of that, laboratory experiments, as the ones addressed in this thesis, are crucial to assess the biological effects of pollutants under different environmental scenarios. Laboratory experiments were performed following similar protocols in order to obtain comparable data, using mussels which were acclimated to constant laboratory conditions in order to avoid the influence of external factors such as temperature and food availability. The experimental variables studied in this thesis were: food availability (Chapter 2), food quality (Chapter 3) and reproduction (Chapter 4), included in the overall concept of 'mussel condition'. In the field study, it was not possible to distinguish between those processes responsible of condition, consequently **mussel nutritive and reproductive states** were jointly considered as **mussel condition**. On the contrary, in laboratory studies we were able to isolate the nutritive from the reproductive state, distinguishing quantitative and qualitative aspects in the context of the nutritive state. In this regard, mussels were laboratory-made depending on the proposed objectives and these "new mussels" were exposed to a contaminant model as it is FLU.

Specific alterations of organisms at cellular or biochemical levels may not be reflected at the individual level, which ultimately reflects health state. For that reason, a battery of biomarker responses at different levels of biological organization (biochemical, immunological and physiological) was considered in this thesis. Moreover, the statistical integration of these biomarkers was performed for a better interpretation of interactions between biomarkers, pollution and biological variables.

1. Biological variability in mussel monitoring programs

Traditionally, large-scale monitoring programs have been designed to avoid mussel biological influence on biomarkers. To do this, mussel sampling is addressed during the mussel resting period according to the JAMP Guidelines for Monitoring Contaminants in Biota (OSPAR Commission, 2010). Following these guidelines "*sampling should take place during late autumn/early winter when mussels are in a more stable physiological state and, in any case, during a period before spawning*". In the OSPAR Region IV, it is considered that mussel gametogenesis takes place between late autumn and winter whereas spawning generally occurs from spring to early summer. However, mussels can present spawning episodes throughout the year (Bayne, 1976). In the Galician Atlantic coast, for instance, two reproductive cycles have been registered, with an important spawning peak in spring and a second and less intense peak in autumn (September-October) (Cáceres-Martínez and Figueras, 1998; 2007). The SMP adopts these guidelines, and the sampling period used for mussel sampling is November-December.

The field study included, for the first time, a comprehensive analysis of biological characteristics of the sampled mussels. Since the study was carried out in November 2012, mussels at the beginning of gametogenesis would be expected. However, the results obtained evidenced that the 23 mussel populations differed both in nutritive condition (CI from 8.7±1.3 to 14.1±1.3) and gonadal development (SMI from 0 to 4). This great variability in mussel condition was observed in previous studies carried out in this area (Albentosa *et al.*, 2012; Bellas *et al.*, 2014). Besides the variation in mussel condition indices (mussel nutritive state), environmental differences between sampling sites showed also a wide range of reproductive state. As described in this thesis, mussels can be *opportunistic species* (Bayne, 1976) in such a way that when food availability conditions are optimal, gametogenesis takes place regardless of the season. This fact was validated in the field study where mussels from more productive coastal areas (Atlantic waters) displayed higher gonadal development.

According to the JAMP (OSPAR Commission 2008), biological effects can be used to indicate the presence of substances, or combination of substances, of concern in terms of decrease of environmental quality. The applicability of biomarkers on marine pollution monitoring programs is conditioned by the capability to discriminate mussel pollutant-responses from the influence of variability in natural processes (Thain *et al.*, 2008). The fact that strong differences in mussel biological parameters coexist at the same time in a large-scale monitoring programs, affects distinct biomarker responses just for the fact that mussels are in a different nutritive and/or reproductive states. This evidence represents a limitation for the adequate interpretation of biomarker responses to pollution. Thus, variability in mussel condition and its influence on biomarker responses, used as indicators of pollution in monitoring programs, need to be studied and considered mainly in high-productively areas with warm weather, as the one studied in this thesis.

2. Biomarker responses to mussel condition

2.1 Immune biomarkers

Hemolymph plays a number of important roles in bivalve physiology. Since hemolymph is associated to gas exchange, osmoregulation, nutrient distribution, waste elimination and internal defense (Gosling, 2003), the immune function might change in relation to changes in mussel condition. In our study, this issue was addressed only when mussels were conditioned to two different quality diets, in the experiment carried out at the LEMAR laboratory during a training research stay as a part of my doctoral formation.

Despite different quality fed mussels were varied in terms of their fatty acid profiles, changes in total biochemical components or in condition indices were not observed. As a result, no important differences were observed in mussel immune parameters. Only one of the five immunological parameters studied showed an effect of the quality of the diet. It was the percentage of dead hemocytes. But, this effect was not of importance. Nevertheless, previous published studies have described changes in the immune parameters of bivalves in relation to microalgal diets (Hégaret *et al.*, 2004) or mono-algae diets supplemented with lipid emulsions (Delaporte *et al.*, 2007). Since, in this thesis, mussel immune parameters were not considered in relation to other mussel conditions: food quantity or reproductive state, it was not possible to provide a conclusive relationship between the effect of mussel condition and inmunology.

2.2 Physiological biomarkers

The most common physiological biomarker used in marine pollution monitoring surveys is scope for growth (SFG), which was included in this thesis as the integration of the main physiological parameters: clearance rate (CR), absorption efficiency (AE) and respiration rate (RR). Tables 26 and 27 summarized the physiological responses obtained in the different experiments performed in this thesis.

The SFG assessment protocol used in this thesis was described by Widdows and Staff (2006) and later modified by Albentosa *et al.* (2012), and is part of the ICES collection of techniques in marine environmental sciences (TIMES), serving as the recommended procedure for marine monitoring programs in OSPAR regions. This technique assumes that when mussels are maintained under ‘near optimal’ laboratory conditions of food and temperature, the measurement of the SFG will be the maximum for that laboratory conditions. Thus, any reduction in the SFG will be caused by the stress produced by the pollutants accumulated in their tissues which are directly related to pollutant environmental concentrations (Goldberg, 1986). Since CR is the physiological parameter with the greatest effect on SFG (Toro *et al.* 2003), all those natural variables affecting CR are also modulating SFG. It is well-known that CR is regulated by particle concentration in such a way that mussels exposed to higher food concentrations displayed the lower clearance rates (Winter, 1978; Widdows, 1978; Bayne and Newell, 1983; Hawkins *et al.*, 2001; Velasco and Navarro, 2003). Results of

our field study showed higher CR in mussels with poorer condition indices. It was hypothesized that mussels with poorer conditions come from habitats with lower food availability. In these areas, mussels respond to this food scarcity increasing its CR to increase food intake. To prove this hypothesis, mussels were conditioned to different food ratios (food quantity experiment) under laboratory conditions. Results showed a gradient of CR was observed according to the nutritive states, being mussels under nutritive stress those which displayed the highest CR values during the SFG test. It is necessary to highlight that CR quantification was obtained with the same standardized food concentration, for all the mussel nutritive conditions. As discussed in Chapter 2, mussels seem to keep an *ecological memory* that comes from their previous feeding conditions, and thus observed differences in CR would result from their past feeding history. In field study, past feeding history was considered natural food availability conditions. However, in the laboratory experiments it was considered the different nutritive conditions from conditioning aquaria. As was suggested by other authors (Bayne 1993; Pérez-Camacho *et al.*, 1995; Iglesias *et al.*, 1996; Labarta *et al.* 1997; Babarro *et al.* 2000; Fernández-Reiriz *et al.*, 2013), this ecological memory is related to physiological adaptations to the particular environmental conditions in the primary habitat (Babarro *et al.*, 2000) or to genetic differentiation (Peterson and Beal, 1989). Since the mussels used in these experiments came from the same population, the differences observed in mussel CR may be attributed to the physiological adaptation to different food ratios prior to the SFG assay. Taking into account that the above mentioned protocol for SFG measurements (Widdows and Staff, 2006) established a period of depuration of 24 h from sampling (at field/laboratory) to measurements, it seems that this period it is not enough to eliminate the effect of mussel condition in CR measurements. This experimental conclusion was supported by the results showed in Chapter 4, where mussels with the same nutritive conditions did not show any differences in their CRs. Interestingly, as observed in Chapter 4 mussel reproductive state did not change the CR pattern whenever the nutritive conditions are equal in both reproductive stages (Table 26).

Table 26. Physiological responses obtained in the different experiments performed in this thesis. CR: Clearance rate and AE: Absorption efficiency.

BIOMARKER	EXPOSURE CONDITION	DESCRIPTION	TOXICANT	TIME OF EXPOSURE	REPRODUCTIVE STATE	NUTRITIVE STATE	MUSSEL CONDITION EFFECT?	FLU EFFECT?	MIX POLLUTANT EFFECT?	WHICH TOXICANT?
CR	Field	Atlantic coast	Mix of pollutants	cronic exposure					+	Hg
		Cantabrian Coast					+			
	Laboratory	Nutritive stressed-mussels (NS-1)	Fluoranthene	short-time exposure 3 weeks	RS-2	NS-1	+			
		Intermediate fed-mussels (NS-2)				NS-2				
		Well fed-mussels (NS-3)				NS-3				
	laboratory	*C.neogracile* conditioning diet	Fluoranthene	short-time exposure 1 week		NS-3				
		H. triquetra conditioning diet								
	Laboratory	Reproductive stage (RS-1)	Fluoranthene	short-time exposure 3 weeks	RS-1	NS-3				
		Resting stage (RS-2)			RS-2			–		
AE	Field	Atlantic coast	Mix of pollutants	cronic exposure			–			Pb
		Cantabrian Coast							+	As
	Laboratory	Nutritive stressed-mussels (NS-1)	Fluoranthene	short-time exposure 3 weeks	RS-2	NS-1				
		Intermediate fed-mussels (NS-2)				NS-2				
		Well fed-mussels (NS-3)				NS-3	–	–		
	laboratory	*C.neogracile* conditioning diet	Fluoranthene	short-time exposure 1 week		NS-3				
		H. triquetra conditioning diet								
	Laboratory	Reproductive stage (RS-1)	Fluoranthene	short-time exposure 3 weeks	RS-1	NS-3				
		Resting stage (RS-2)			RS-2					

Table 27. Physiological responses obtained in the different experiments performed in this thesis. RR: Respiration rates and SFG: Scope for growth.

BIOMARKER	EXPOSURE CONDITION	DESCRIPTION	TOXICANT	TIME OF EXPOSURE	REPRODUCTIVE STATE	NUTRITIVE STATE	MUSSEL CONDITION EFFECT?	FLU EFFECT?	MIX POLLUTANT EFFECT?	WHICH TOXICANT?
RR	Field	Atlantic coast	Mix of pollutants	cronic exposure					+	Cu
									–	Zinc
		Cantabrian Coast					+			
	Laboratory	Nutritive stressed-mussels (NS-1)	Fluoranthene	short-time exposure 3 weeks	RS-2	NS-1	+			
		Intermediate fed-mussels (NS-2)				NS-2		+		
		Well fed-mussels (NS-3)				NS-3		+		
	laboratory	*C.neogracile* conditioning diet	Fluoranthene	short-time exposure 1 week		NS-3				
		H. triquetra conditioning diet								
	Laboratory	Reproductive stage (RS-1)	Fluoranthene	short-time exposure 3 weeks	RS-1	NS-3	+			
		Resting stage (RS-2)			RS-2					
SFG	Field	Atlantic coast	Mix of pollutants	cronic exposure					+	Pb and As
		Cantabrian Coast					+		+	As
	Laboratory	Nutritive stressed-mussels (NS-1)	Fluoranthene	short-time exposure 3 weeks	RS-2	NS-1	+	+		
		Intermediate fed-mussels (NS-2)				NS-2				
		Well fed-mussels (NS-3)				NS-3				
	laboratory	*C.neogracile* conditioning diet	Fluoranthene	short-time exposure 1 week		NS-3				
		H. triquetra conditioning diet								
	Laboratory	Reproductive stage (RS-1)	Fluoranthene	short-time exposure 3 weeks	RS-1	NS-3				
		Resting stage (RS-2)			RS-2			–		

In a similar way, an effect of mussel condition was evidenced on the mussel absorption efficiencies (AE). Differences in mussel absorption efficiencies could be related to both, food quantity and quality. In Chapter 2, mussels with a wide range of nutritive states showed differences in mussel AEs. During the conditioning period to different food ratios, mussels maintained at high food concentration (better nutritive state) were those that showed lower AEs. Conversely, during the SFG assay, the same mussels showed the higher AEs. Although it apparently seems a contradictory result, the same relationship between AE and ingestion was observed in both experimental events (conditioning and SFG assay): AE decreases when ingestion increases. In addition to food availability, different AE have been also described under different food qualities (Chapter 3). In this case, mussels' AE were not registered but, according to other studies where similar diets were supplied to mussels, it is known that diatoms are better digested by mussels than dinoflagellates (Rouillon and Navarro, 2003). Contrary to mussel nutritive state, no significant effect of reproductive state was observed in AE (Chapter 4, Table 26). Mussels were fed with the same ratio and kept under the same standardized laboratory conditions at two reproductive stages, which could promote a similar AE pattern. Since food absorption depends on both, feeding rate and efficiency of food processing (Gosling, 2003), and considering that CR is strongly affected by the past feeding history, both CR and AE could be affected in the SFG test during monitoring programs where mussels came from different trophic scenarios.

Regarding mussel respiration, this physiological parameter was also affected by mussels' condition. In this thesis, mussels under nutritive stress displayed the highest RR. It was a contradictory result considering the pattern of mussel respiration rates previously published. In a previous study (Griffiths and Griffiths, 1987), higher respiration rates were related to the high feeding activity, which promotes a high metabolic cost related to the feeding effort. This apparently contradiction can be explained by mussel feeding behavior. In our study, nutritive-stressed mussels were those that showed the higher feeding rates during the SFG assay. A similar pattern of mussel RR dependence on endogenous factors was observed in Chapter 4, in which mussels at the reproductive state showed higher RRs compared with mussels at the resting state. This relationship between gametogenesis, body reserves and routine rate of oxygen consumption has also been described by Bayne and Thompson (1970), Widdows and Bayne (1971) and Bayne (1973), as was previously discussed in Chapter 4.

The integration of all these physiological parameters in the SFG was also affected by mussel condition, as a direct consequence of the effect of mussel condition on the individual rates (CR, AE, RR). This effect observed under laboratory conditions, supports previous findings in monitoring programs carried out by our research group (Albentosa *et al.*, 2012; Bellas *et al.*, 2014; Chapter 1), in which a negative correlation between SFG and mussel condition was suggested. In Chapter 2, mussels with the lower nutritive state displayed the highest SFG, as a result of the higher CR observed in this

mussel condition. The strong effect of mussel condition on CR, and therefore on SFG, represents a serious drawback for the use of this biomarker in monitoring programs. On the other hand, although mussels showed higher RR at the reproductive stage, this was not reflected in a significantly reduction in the SFG, since the more decisive CR and AE parameters did not differ between reproductive states.

2.3 Biochemical biomarkers

In the same way as physiological parameters, the effect of mussel condition on biochemical parameters was tested in this thesis. Laboratory experiments presented consistent results indicated by the observed repetitiveness. Tables 28, 29 and 30 shows a summary of the biochemical biomarkers results obtained in this thesis. Considering antioxidant responses, SOD, CAT and GPx resulted affected by nutritive (Chapter 2) and reproductive stressful conditions (Chapter 4), whereas SOD, CAT and GR resulted affected by nutritive quality conditions (Chapter 3). These results suggest that antioxidant activities are strongly susceptible to changes in mussel biological states, and this should be taken into account when these biomarkers are tested in laboratory experiments but, above all, in field studies, such as those carried out within large scale monitoring programs where a great variability of mussel conditions coexists at the same time.

Table 28. Biochemical biomarker responses obtained in the different experiments performed in this thesis. SOD: superoxide dismutase and CAT: Catalase

BIOMARKER	EXPOSURE CONDITION	DESCRIPTION	TOXICANT	TIME OF EXPOSURE	REPRODUCTIVE STATE	NUTRITIVE STATE	MUSSEL CONDITION EFFECT?	FLU EFFECT?	MIX POLLUTANT EFFECT?	WHICH TOXICANT?
SOD	Field	Atlantic coast	Mix of pollutants	cronic exposure						
		Cantabrian Coast								
	Laboratory	Nutritive stressed-mussels (NS-1)	Fluoranthene	short-time exposure 3 weeks	RS-2	NS-1	+			
		Intermediate fed-mussels (NS-2)				NS-2				
		Well fed-mussels (NS-3)				NS-3				
	laboratory	*C.neogracile* conditioning diet	Fluoranthene	short-time exposure 1 week		NS-3	+	+		
		H. triquetra conditioning diet								
	Laboratory	Reproductive stage (RS-1)	Fluoranthene	short-time exposure 3 weeks	RS-1	NS-3	+			
		Resting stage (RS-2)			RS-2					
CAT	Field	Atlantic coast	Mix of pollutants	cronic exposure					+	Metals (As)
		Cantabrian Coast					+		+	Cu
	Laboratory	Nutritive stressed-mussels (NS-1)	Fluoranthene	short-time exposure 3 weeks	RS-2	NS-1	+			
		Intermediate fed-mussels (NS-2)				NS-2		+		
		Well fed-mussels (NS-3)				NS-3				
	laboratory	*C.neogracile* conditioning diet	Fluoranthene	short-time exposure 1 week		NS-3	+	+		
		H. triquetra conditioning diet								
	Laboratory	Reproductive stage (RS-1)	Fluoranthene	short-time exposure 3 weeks	RS-1	NS-3	+			
		Resting stage (RS-2)			RS-2			+		

Table 29. Biochemical biomarker responses obtained in the different experiments performed in this thesis. GPx: Glutathione peroxidase and GR: Glutathione reductase.

BIOMARKER	EXPOSURE CONDITION	DESCRIPTION	TOXICANT	TIME OF EXPOSURE	REPRODUCTIVE STATE	NUTRITIVE STATE	MUSSEL CONDITION EFFECT?	FLU EFFECT?	MIX POLLUTANT EFFECT?	WHICH TOXICANT?
GPx	Field	Atlantic coast	Mix of pollutants	cronic exposure						
		Cantabrian Coast								
	Laboratory	Nutritive stressed-mussels (NS-1)	Fluoranthene	short-time exposure 3 weeks	RS-2	NS-1	+			
		Intermediate fed-mussels (NS-2)				NS-2				
		Well fed-mussels (NS-3)				NS-3		+		
	laboratory	*C.neogracile* conditioning diet	Fluoranthene	short-time exposure 1 week		NS-3				
		H. triquetra conditioning diet								
	Laboratory	Reproductive stage (RS-1)	Fluoranthene	short-time exposure 3 weeks	RS-1	NS-3	+			
		Resting stage (RS-2)			RS-2			+		
GR	Field	Atlantic coast	Mix of pollutants	cronic exposure					+	Cd, Zinc and Chlordanes
		Cantabrian Coast					+		+	AS
	Laboratory	Nutritive stressed-mussels (NS-1)	Fluoranthene	short-time exposure 3 weeks	RS-2	NS-1				
		Intermediate fed-mussels (NS-2)				NS-2				
		Well fed-mussels (NS-3)				NS-3		+		
	laboratory	*C.neogracile* conditioning diet	Fluoranthene	short-time exposure 1 week		NS-3	+	+		
		H. triquetra conditioning diet								
	Laboratory	Reproductive stage (RS-1)	Fluoranthene	short-time exposure 3 weeks	RS-1	NS-3				
		Resting stage (RS-2)			RS-2					

Table 30. Biochemical biomarker responses obtained in the different experiments performed in this thesis. GST: Glutathione-S-transferase and LPO: lipid membrane peroxidation.

BIOMARKER	EXPOSURE CONDITION	DESCRIPTION	TOXICANT	TIME OF EXPOSURE	REPRODUCTIVE STATE	NUTRITIVE STATE	MUSSEL CONDITION EFFECT?	FLU EFFECT?	MIX POLLUTANT EFFECT?	WHICH TOXICANT?
GST	Field	Atlantic coast	Mix of pollutants	cronic exposure						
		Cantabrian Coast					+		+	Cu and HCHs
	Laboratory	Nutritive stressed-mussels (NS-1)	Fluoranthene	short-time exposure 3 weeks	RS-2	NS-1		–		
		Intermediate fed-mussels (NS-2)				NS-2				
		Well fed-mussels (NS-3)				NS-3	–	+		
	laboratory	*C.neogracile* conditioning diet	Fluoranthene	short-time exposure 1 week		NS-3	+			
		H. triquetra conditioning diet								
	Laboratory	Reproductive stage (RS-1)	Fluoranthene	short-time exposure 3 weeks	RS-1	NS-3				
		Resting stage (RS-2)			RS-2					
LPO	Field	Atlantic coast	Mix of pollutants	cronic exposure			+			
		Cantabrian Coast								
	Laboratory	Nutritive stressed-mussels (NS-1)	Fluoranthene	short-time exposure 3 weeks	RS-2	NS-1				
		Intermediate fed-mussels (NS-2)				NS-2				
		Well fed-mussels (NS-3)				NS-3				
	laboratory	*C.neogracile* conditioning diet	Fluoranthene	short-time exposure 1 week		NS-3				
		H. triquetra conditioning diet								
	Laboratory	Reproductive stage (RS-1)	Fluoranthene	short-time exposure 3 weeks	RS-1	NS-3				
		Resting stage (RS-2)			RS-2					

In the field study, an inverse relationship between the antioxidant responses and mussel nutritive state was reported in the Cantabrian coast, which has been described as a region with low food availability, in agreement with recent studies carried out in this area (Bellas *et al.*, 2014). Moreover, laboratory experiments confirmed that mussels with lower mussel condition displayed the highest antioxidant activities (SOD, CAT, GR) and GST, in accordance to previous works in which adverse nutritive or fasting conditions produced degradation of endogenous biochemical components (Ansaldo *et al.*, 2007), promoting the activation of the autophagy system. This activation involves an increase of the number and activity of lysosomes (Moore, 2004; Moore *et al.*, 2007) which are sites of ROS generation (Moore *et al.*, 2007).

Similar results were obtained in mussels exposed to different quality dietary conditions. Mussels fed with diatoms displayed higher values of antioxidant activities. Despite the fact that mussels were fed with the same ratio of diatoms and dinoflagellates, differences in antioxidant enzymes activity suggest that not only quantity but also quality of diet is an important factor to take into account in the evaluation of biomarker responses. In this thesis, the higher lipid content supplied by the diatom dietary condition might promote the activation of different metabolic pathways and change the antioxidant activities, as opposed to the dinoflagellate dietary condition. Our results were in agreement with previous studies carried out with fish in which an enrichment of lipids, specifically PUFAs, elicit an increase of ROS production and, consequently higher antioxidant activities (SOD and CAT) (Rueda-Jasso *et al.*, 2004) and increased lipid peroxidation (Olsen and Henderson, 1997; Zeng *et al.*, 2015).

SOD, CAT and GPx activities were also affected by mussel reproductive state, greater at the reproductive stage. The increase of mussel metabolic rate attributed to gametogenesis was reported previously by Widdows and Bayne (1971) and Bayne (1973). High investment in reproduction can generate a trade-off in the allocation of energy, leading to the detriment of important physiological functions. The energy demand is satisfied by an increase in the metabolic rate during the reproductive period (Angilleta and Sears, 2000), which promotes an increase in intracellular respiration and is reflected in an elevation of ROS production (Béguel *et al.*, 2013). Conversely, GST did not show any significant effect of reproductive state. It is known that antioxidant enzymatic activities are more subjected to seasonal control than other detoxification enzymes. In fact, previous studies found no seasonal variation in GST activities in mussels (Power and Sheehan, 1996; Vidal-Liñán *et al.*, 2010). In addition, a smooth pattern of geographical variability was observed in mussels' GST activity during a monitoring survey in the same study area as this thesis (Bellas *et al.*, 2014), even when neighbor sites may present large differences in chemical pollution. It seems that GST is more stable than antioxidant enzymatic activities; nevertheless, considering that GST was also affected by nutritive stress and that GST response to pollution is dependent on the nutritive condition (Chapter 2), it is also necessary to consider mussel biological state for a correct interpretation of this biomarker.

Mussel condition, understood as both nutritive and reproductive states, did not affect LPO activity probably because the induction of antioxidant enzymes provided protection against lipid membrane peroxidation (Campillo *et al.*, 2013; Fernández *et al.*, 2010, 2012; González-Fernández *et al.*, 2015).

3. Biomarker responses to pollution under several mussel conditions

In this thesis, FLU was used as a model pollutant for the laboratory experiments in order to simplify the biomarker responses to pollution in mussels with different endogenous conditions related to its nutritive (un terms of quantity and quality) and reproductive state. Effect of FLU exposition on all the biomarkers studied has been summarized in the above detailed Tables from 26 to 30.

3.1. Bioaccumulation

The interest in using organisms to monitor contaminant levels relies on the fact that sporadic contamination may not be recorded in the water while aquatic organisms can accumulate those substances providing an integrated record of contaminant levels. It is generally accepted that the assessment of marine environmental quality is better addressed on the basis of the integration of chemical and biological measurements. In fact, the European Marine Strategy Framework Directive (MSFD, 2008/56/EC) advocates an approach based on chemical and biological monitoring for the assessment of pollution in marine ecosystems. In this sense, it has been registered greater accumulative capacity in mussels than in other marine invertebrates, which have been used worldwide as sentinels in pollution monitoring programs (Livingstone, 1991).

The bioavailability of different contaminants in the marine environment will depend, to varying extents, on factors such as salinity, turbidity, dissolved organic matter, particulate organic matter, particle size distribution, organic content or redox conditions, among others. In this thesis, a great spatial variability of pollutants concentration in mussel tissues has been reported under natural environmental conditions, ranging from 13.25 to 1015 µg Kg^{-1} in PAHs as an example (Chapter 1). This variability was attributed to differences in the anthropogenic sources of pollution. However, differences in toxicant bioaccumulation were also observed in laboratory experiments, in relation to mussel nutritive conditions (Chapter 2). Table 31 shows the accumulation efficiencies in mussels exposed to FLU under the three experimental scenarios. In general, mussels with better nutritive condition displayed lower accumulation values. This was discussed according to previous studies carried out with trace metals, in which higher mussel´s tissue weight (higher condition indices) implies lower accumulation of metals (Mubiana *et al.*, 2006; Richir and Gobert, 2014; Zhong *et al.*, 2013). In the referred studies, it was considered that pollutants were ‘diluted’ in the higher biomass of mussels with higher condition indices. However, in present experiments toxicant was exposed at equal amount related to the mussel biomass,

consequently the mentioned dilution explanation is not suitable. As an alternative, differential bioaccumulation could be attributed to lower food absorption rates of mussels fed at high food ratio. The hypothesis that lower absorption efficiencies promote lower pollutant accumulation has been documented by other authors (Wang and Wong, 2003) who described that an increase of algae concentration resulted in a decrease in assimilation efficiency of some metals (Cd, Se and Zn). It is necessary to highlight at this point that FLU concentration registered in mussel tissues was high, regardless of the significant differences between conditions.

Regarding food quality conditions, significant differences in accumulation efficiencies were registered in mussels fed with the two algae species (Chapter 3). Mussels fed with diatoms showed double accumulation efficiency that those fed with dinoflagellates. A better absorption efficiency of diatoms than dinoflagellates has been described in mussels, as a consequence of the easier digestibility associated to the diatoms' structure (Rouillon and Navarro, 2003) which can explain the higher bioaccumulation of diatom-fed mussels. Other hypothesis points to the cell size difference between both microalgae. Due to the high volume of *H. triquetra* cells, higher number of *C. neogracile* cells was necessary to obtain the same food ratio with both algal species. The higher contact surface in the diatom diet could have provided a higher pollutant adsorption and consequently higher bioaccumulation. Moreover, diatom cells showed higher lipid content which might be responsible for the higher accumulation of FLU, which is a lipophilic compound with high affinity for biological compartments with high lipid content. Therefore, not only food availability but also food quality can have an effect on the bioaccumulation of FLU. This effect has been recently studied, and has been found that the dietary pathway is an important contributor to the global accumulation of metals (Hédouin *et al.*, 2010) and organic contaminants such as PAHs (Croxton *et al.*, 2012) in marine organisms. Such findings evidence that these factors should be considered for the correct implementation of chemical analysis in marine pollution monitoring programs. Guidelines including some of these aspects were actually considered by the OSPAR Convention "*trophic* state *and feeding preferences will strongly affect the relative impact of sediment-associated metals on different benthic organisms"* (OSPAR, 2010) but, due to the high cost of their implementation, they are not commonly performed.

Table 31. FLU accumulation efficiency (ACE) of mussels under different experimental conditions.

Factor	Experimental conditions	% ACE
Food availability	Nutritive stressed-mussels (NS-1)	8.8
	Intermediate fed-mussels (NS-2)	11.1
	Well fed-mussels (NS-3)	6.7
Food quality	*C.neogracile* dietary conditioning	53.4
	H. triquetra dietary conditioning	20.2
Reproduction	Reproductive state (RS-1)	15.4
	Resting state (RS-2)	31.9

The reproductive state has also been found to influence bioaccumulation of toxicant in mussels which achieved higher pollutant concentration during resting time (Chapter 4). Taken into consideration that all reproductive stages may coexist at the same time in a large-scale mussel monitoring program, differences in accumulation may be related to this factor. In this sense, differences in pollutant concentrations in field studies could be related to the different reproductive conditions between sampling sites. FLU bioaccumulation efficiencies registered in mussels at the reproductive stage were similar to those obtained in the food availability study (Chapter 2). However, efficiencies registered during the resting state were double than those obtained in food quantity experiment although in this last case, mussels were also in a resting condition (Table 31). Bioaccumulation differences could be due to differences in the precise time of the gametogenic cycle as both were carried out at different times of the year (summer in food availability and autumn in resting state experiments). In any case, mussels exposed to high FLU concentrations during the resting time, showed higher FLU concentrations and accumulation efficiencies than mussels at the reproductive state. Differences observed in mussel accumulation could be caused by the high energetic cost of reproduction that might increase the combustion of polluted assimilated food. In addition, as mentioned before, FLU is a highly hydrophobic compound which tends to bind to the non-polar components of mussels, as lipids, especially to neutral lipids (Jorgensen *et al.*, 1997), which constitute most egg reserves in marine bivalves (Bayne *et al.*, 1975). Mussels exposed to FLU at the reproductive stage evidenced a detriment of their reproductive tissue, including symptoms of partial spawning; in fact, a reduction of around 40% of mussel *reproductive potential* was quantified at high exposure compared to control, being this fact responsible of the lower FLU concentration observed in reproductive mussels. In *M. edulis* exposed to cadmium a stimulating effect of the metal to spawning was described (Kluytmans *et al.*, 1988). Seasonal differences in pollutant bioaccumulation have been long recognized and, for instance, have been reported in field mussel populations exposed to PCBs (Hummel *et al.*, 1989, 1990) or to PAHs (Bourgeault and Gourlay-Francé, 2013). In these studies, pollutant concentrations varied significantly between seasons, with minimum values obtained during gametogenesis and post-spawning, and the maximum values reached during the period of sexual rest and pre-spawn.

To conclude, the most traditional view to assess marine pollution, which is the measurement of pollutant concentrations in biota is clearly compromised under the light of the results obtained in all the experiments of this thesis. We can´t consider that bioaccumulation in biota is directly related to environmental pollutant concentrations as this process is clearly affected by several biological variables (nutritive and reproductive conditions).

3.2 Immune biomarkers

Immune responses have been considered as indicators of mussel health state, therefore they may lead to a better understanding of the effect of pollutants in these organisms. Although mussels seem to be less sensible to pollution than other bivalve species (Dyrynda *et al.*, 2000), in this thesis we observed a significant effect of the toxicant on the mussel immune parameters studied, at environmentally relevant concentrations. Regardless of dietary conditioning, a positive correlation was observed between FLU accumulated and phagocytosis or hemocyte ROS production. Even though a general effect was observed in immune responses, they were also dependent of dietary conditioning. There was a significant increase of phagocytosis capacity and hemocyte ROS production with toxicant exposure in mussels fed with the diatom *C. neogracile*, whereas no effect was observed in mussels fed with the dinoflagellate *H. triquetra*. These results could be a direct consequence of the higher toxicant concentration accumulated in mussels fed with diatoms which was twice the observed in mussels fed with the dinoflagellates, as discussed above. This was supported by previous studies in which PAH compounds were highly adsorbed to cells in a diatom culture (*Nitzschia brevirostris*) (Croxton *et al.*, 2012). The toxicant concentration used in our study (30 µg FLU L^{-1}) was lower than those used in previous studies of immune responses in mussels (Guinnapas *et al.*, 2012; Coles *et al.* 1994). Moreover, in these studies not only the toxicant concentration was higher but also a mix of contaminants was used, such as a cocktail of PAHs. Despite the lower toxicant concentration which was used in this thesis, the immune parameters provided an early response to toxicant exposure, suggesting that these responses could be considered as useful tools in pollution studies.

3.3 Physiological biomarkers

The observed variability of physiological responses according to mussel condition, suggests that interferences in the establishment of relationships between SFG effects and pollution will arise in field studies, as this thesis evidenced (interaction in the two-way ANOVA between mussel condition and toxicant in Chapter 2; $p<0.05$ and Chapter 4; $p<0.001$).

Previous studies have postulated that the CR is the physiological parameter most affected by pollution (Al-Subiai *et al.*, 2012; Beiras *et al.*, 2012; Toro *et al.*, 2003). In this thesis, only a negative effect of toxicant on the CR was registered in mussels at the resting stage (Chapter 4). On the contrary, mussels that showed also a low gametogenic development in the food quantity experiment, and fed on the same ratio, did not show the same negative effect in CR. A possible explanation could be that mussels at resting time accumulate 2-fold higher quantities of toxicant in their tissues than mussels from the food quantity experiment (Chapter 2). Moreover, although the amount of FLU supplied per mussel weight, food ratio, temperature and time of exposure were similar in both experiments, differences in the time of the general annual cycle could be partially responsible for the distinct physiological responses observed. While in Chapter

4 mussels were sampled in autumn, in Chapter 2, mussels were sampled and conditioned until spring. Despite mussels were not in the reproductive stage, the period of the year could determine the start of mobilization of energetic reserves, altering mussels' responses to pollution.

On the other hand, food absorption was only affected by toxicant, when mussels at high nutritive conditions (NS-3, well-fed) evidenced a reduction of their AE higher than 50% at high toxicant concentration during the SFG test. In mussels, many authors have reported that PAHs exposure can induce pathologic changes in the digestive gland and in the lysosomal membrane stability (Lowe *et al.*, 1981; Toro *et al.*, 2003, Porte *et al.*, 2001a; Solé *et al.*, 1995) which could promote a reduction of AE. In this thesis, not only AE but also RR resulted affected by the toxicant (Chapter 2). An increase of mussels' RR was observed in the presence of toxicant in mussels with good nutritive state: NS-2 (intermediate-fed) and NS-3 (well-fed) mussels, at high toxicant concentration. The increase in the energy lost through respiration after toxicant exposure has been previously reported by several authors (Widdows *et al.*, 1982; Donkin *et al.*, 1989; Widdows and Donkin., 1991; Toro *et al.*, 2003b). The effect observed in RR and AE was not evident in mussels from the reproduction study (Chapter 4), so it was not possible to establish a clear pattern in the effect of FLU on these physiological parameters.

Finally, we analyzed the effect of toxicant on the SFG, which is one of the most important physiological parameters used in pollution monitoring programs. In this context, several authors have described a negative correlation between pollution and SFG under field (Widdows *et al.*, 1995; 2002) and laboratory (Widdows *et al.*, 1982; 1984; Donkin *et al.*, 1989; Widdows and Donkin, 1991; Eertman *et al.*, 1995) conditions. Two main conclusions may be drawn from the experiments performed in this thesis in which physiological biomarkers where tested (Chapter 2 and 4). First, SFG were negatively affected by the toxicant only when this pollutant had an effect on the CR (Chapter 4). This conclusion is in agreement with previous studies which described a negative correlation between SFG and PAH (Donkin *et al.*, 1989; Eertman *et al.*, 1995; Widdows and Donkin, 1991; Widdows *et al.*, 1995; 2002), and with those suggesting that CR is the parameter most affected by pollution (Al-Subiai *et al.*, 2012; Beiras *et al.*, 2012; Toro *et al.*, 2003). Nevertheless, immature mussels with similar nutritive conditions (food quantity experiment) as those of resting mussels in the reproductive experiment did not show the same negative effect in SFG (Table 27). In this sense, it is essential to understand the interaction of pollution and mussel condition with physiological parameters. The increase of the SFG observed in nutritive stressed mussels not only in control mussels but also in exposed mussels, provide a positive relationship between toxicant and SFG (Chapter 2). These results are contradictory to previous studies which evidenced a negative correlation between SFG and PAHs (cited above), but are in agreement to the field study conducted here (Table 27), where a positive correlation between SFG and pollution ("Pb+As" in the Atlantic coast and "As" in the Cantabrian coast), was observed.

To conclude, this thesis has shown that SFG is a biomarker highly sensitive to mussel nutritive condition and not as sensitive to pollutants, at least to PAHs. Under extreme pollution conditions or under particular mussel biological conditions, physiological measurements could reflect a direct effect of pollution (Chapter 4) but, since different mussel conditions are present concurrently, the coexistence of two powerful physiological stressors, as nutritive and pollution, makes the identification of the physiological response to pollution really complex.

3.4 Biochemical biomarkers

A summary of the effects of the toxicant on biochemical biomarkers, is shown in Tables 28-30, but only a positive or negative effect of FLU was considered, regardless of the FLU concentration used in each experiment (3 µg L^{-1}, 30µg L^{-1} and 60µg L^{-1}), in order to simplify results. As it can be appreciated, biomarker responses to FLU were different depending on the experimental conditions.

The first point to notice is that all biomarkers considered, with the exception of LPO, resulted affected by FLU. In previous studies, the increase in the antioxidant enzymes activity in polluted areas was accompanied by no significant differences in MDA concentration, reflecting an adaptation to the contaminant exposure (Box *et al.*, 2007, Cheung *et al.*, 2001), or even a inverse relationship between antioxidant responses and LPO, suggesting a protector role of antioxidant biomarkers (Campillo *et al.*, 2013; Fernández *et al.*, 2010; González-Fernández *et al.*, 2015). Antioxidant enzymes, such as SOD, CAT and GPx, are direct ROS scavenging antioxidant enzymes and, therefore, are the first enzymes activated in presence of pollution (Regoli and Giuliani, 2014), consequently, the activation of this enzymatic responses under pollution stress is expected.

On the other hand, and in the same way as reported for physiological biomarkers, biochemical biomarker responses were dependent on the metabolic condition of mussels. Mussels with better nutritive condition (NS-2 and NS-3) were affected by toxicant exposure, evidencing higher values of CAT, GR, GPx and GST in presence of FLU (Chapter 2). In the same way, mussels with better food absorption efficiencies (those fed with diatoms) evidenced higher values of SOD, CAT and GR in presence of FLU (Chapter 3), and mussels at gonadal resting state displayed the higher values of CAT and GPx in the presence of FLU (Chapter 4).

At this point, two important factors that could be influencing the different responses observed may be highlighted. First, intra-group statistical analyses were performed, that means that each experiment was analyzed separately. In this sense, as discussed above, mussels with the highest values of toxicant accumulated in their tissues were also those presenting an induction of the biochemical biomarkers, in line with the increase of ROS production and antioxidant response in mollusks exposed to PAHs reported in the literature (Solé *et al.*, 1995; Porte el al., 2001a; Fernandez *et al.*, 2010). Specifically, some PAHs such as FLU, in addition to binding with the biomolecules, have the ability to produce ROS resulting in induction of oxidative

damage (Al-Subiai *et al.*, 2012; Coles *et al.*, 1994; Lowe *et al.*, 1995). Second, and more important within the context of this thesis, is that those biochemical biomarkers affected by FLU were, in most cases, also affected by mussel nutritive condition and/or reproductive state. In fact, the interaction of both factors and pollution with the biomarker responses pointed out that the effect of mussel nutritive state and the effect of the reproductive state on the biochemical biomarkers was stronger than the effect of the toxicant (Chapters 2, 4), and would be masking the response to pollution, as suggested, for instance by the IBR integration. This information is critical in field studies, mainly in large-scale monitoring programs, were food conditions are highly variable between sampling sites. Results from this thesis evidenced, that biochemical biomarkers responses are affected not only by pollutant concentration in mussel tissues but also by mussel condition and reproductive state. Therefore, mussel condition and reproductive state should be considered as additional variables in marine pollution monitoring programs in order to better understand the changes observed in biomarker responses.

CONCLUSION

Effect of confounding factors in biological responses of mussels, *Mytilus galloprovincialis*, to pollution: Implication in large-scale monitoring programs

Carmen González Fernández, 2016

CONCLUSIONS

Biomarker responses in Marine Monitoring Programs

1. A ***great geographical variability*** was observed in the biology of mussel populations used for the assessment of pollution. High variability was observed especially on the *mantle development* and on the *carbohydrates content*, which were related to both nutrition and reproduction of the mussel. The larger the area sampled by a monitoring program, the higher the variability of biological parameters observed, which is related to the natural variability of environmental factors, mainly food availability.

2. Two well differentiated patterns in the response of biomarkers to ***pollution*** and to ***mussel biological variability*** were observed: no significant relationship between biomarker responses and biological variables was found in the Atlantic area, whereas a significant influence of mussel biology on biomarkers was observed, in the Cantabrian area, where biomarkers were not related to pollution. This result was explained by the effect of the biological parameters studied, which acted as *confounding factors* modifying the expected biomarker responses to pollution.

3. Marine Monitoring Programs should include the ***measurement of biological and environmental parameters*** for a correct interpretation of biomarkers responses to pollution, being this recommendation crucial in large-scale monitoring programs covering highly variable ecosystems.

Biomarker responses to Mussel Condition

4. Mussel nutritive state, in terms of food quality, had no effect on the ***immune biomarkers.*** Mussel immune parameters were not assayed under other mussel conditions, thus, it was not possible to provide a conclusive relationship between mussel condition and immunology.

5. The ***biomarker SFG*** was highly dependent on mussel *past-feeding history*. This fact compromises the use of the SFG in large-scale monitoring programs, which are characterized by a great variability in trophic conditions between sites and between sampling periods. All the parameters integrated in the SFG (***feeding, absorption*** and ***respiration***) were strongly dependent on the mussel nutritive condition, which was directly related to their past-feeding history (environmental food availability) previous to the measurements, although these measurements are made under controlled laboratory conditions, with the same food concentration. Conversely, mussel reproductive state did not affect SFG biomarker.

6. The level of the ***biochemical biomarkers*** in the digestive gland of mussels (*C**AT, SOD, GPx*** and ***GR***) were strongly affected by food quality and quantity, and also by mussel reproductive state. Since antioxidant enzymes were highly dependent on mussel condition, they are not considered alone as good indicators of pollution in monitoring programs. Unlike the antioxidant activities, ***LPO*** was not affected by the experimental variables considered: food quantity and quality, or reproduction. ***GST*** did not show a clear relationship with mussel condition.

Biomarker responses to Pollution under several Mussel Conditions

7. ***Bioaccumulation*** was dependent on the mussel condition state. The pollutant showed higher accumulation by mussels when they were fed with better digestibility or higher lipid content dietary conditions. Moreover, immature mussels showed higher pollutant accumulation than reproductive organisms, which showed a more efficient detoxification system. As a consequence, pollutants are not accumulated in biota only as a function of their environmental concentrations, but also depending on mussel condition (nutrition and reproduction).

8. The responses of ***immune biomarkers*** were directly associated to pollutant exposure. Higher immune responses were associated with the highest pollutant accumulation observed in mussels fed with *C.neogracile*. Thus, the different immune response observed depending on mussels dietary conditions suggested that diet quality acted as a *confounding factor* also masking the response of immune parameters to pollution.

9. The effect of mussel nutrition on the ***SFG biomarker*** was higher than the exposure to pollutants. This was caused by the strong and persistent effect of food availability on the filtration rates before the SFG measurement. In the same way, the SFG response to pollution was dependent of mussel reproductive state. A negative effect on SFG was observed in resting mussels in presence of pollution. These results were promoted by the higher toxicant accumulation efficiency evidenced by resting mussels. Considering the great variability of nutritive and reproductive states observed in large-scale monitoring programs, further research is needed to assess the use of this biomarker as an indicator of pollution in these programs.

10. The responses of ***biochemical biomarkers*** to pollutant exposure were different depending on mussel condition (both nutritive and reproductive state). The biological changes induced during specific experimental conditions, as nutritive stress or reproduction effort, increased antioxidant activities. This promotes antioxidant protection, decreasing other pollutant effects such as bioaccumulation. Due to the evidenced relationship between mussel condition and antioxidant activities, this responses alone are not considered good indicators of pollution in monitoring programs. Although ***LPO*** was the biochemical biomarker less affected by mussel condition, it was

not susceptible to the toxicant exposure assayed in this thesis (FLU). In addition, as commented above, ***GST*** response to pollution was different depending on mussel condition (both, nutritive and reproductive state) being not possible to establish a clear pattern.

11. Mussels have been classified as highly-resistant animals to pollution. This is why they have a wide geographical distribution occupying highly polluted areas. At the same time, they are intertidal animals and, as a consequence, they are used to highly variable environmental conditions, and have developed complex physiological mechanisms to maximize energy intake. These regulatory processes make mussels very sensitive to food availability at all biological levels, from cell biochemistry to individual physiology. They are opportunistic species that are able to switch metabolic pathways towards gametogenesis when food conditions are favorable. The response of biomarkers is strongly related to biological processes: energy balance, reproduction or nutritive state and, as a consequence, pollution is not the only variable affecting biomarkers responses. Thus, it is necessary to consider ***mussel condition*** as an important variable to take into account in monitoring programs, for the proper interpretation of biomarker responses to pollution.

RESUMEN

Factores de confusión en la respuesta biológica del mejillón, *Mytilus galloprovincialis*, a la contaminación: Optimización de los protocolos de monitorización biológica

Carmen González Fernández, 2016

INTRODUCCIÓN

Los ecosistemas marinos son, en última instancia, los receptores de gran cantidad de compuestos químicos. Estos compuestos tienen, en su mayoría, un origen natural, pero el uso de ciertas sustancias en actividades antrópicas tales como la minería, industria o la agricultura, ha propiciado una producción masiva y, como consecuencia, la liberación de estos compuestos en el medio marino. La introducción de estas sustancias químicas, denominadas ***contaminantes***, que tienen un potencial tóxico para el medio marino o pueden producir un deterioro del mismo, se conoce como ***contaminación marina***.

Los contaminantes pueden llegar al medio marino desde diversas fuentes: por vía fluvial, vía atmosférica o a través de vertidos directos de forma planificada o accidental Una vez introducidos en el medio, los contaminantes pueden interactuar con los diversos compartimentos ambientales: agua, sedimento y biota. Dependiendo de las características de los contaminantes, estos, pueden quedar libres en la columna de agua, o asociarse a partículas de sedimento y materia orgánica. En ambos casos, los seres vivos están potencialmente expuestos a la contaminación de forma directa, ya que están en contacto con los contaminantes la columna de agua, pero también de forma indirecta, debido a que la mayoría de los organismos marinos se alimentan de la materia orgánica u otros seres vivos que han estado previamente expuestos a la contaminación. Este hecho, favorece la acumulación de contaminantes en los tejidos de los organismos (***bioacumulación***), siendo posible la transferencia de contaminantes a lo largo de la cadena trófica.

La continua emisión de contaminantes al medio natural y el riesgo potencial que genera, tanto para los organismos marinos como para la salud humana, ha favorecido la creación de una serie de acuerdos internacionales y regionales con el objetivo de evaluar y controlar la contaminación en el medio marino. En el ámbito de las aguas costeras españolas se encuentran, el Convenio OSPAR para el control de la contaminación en la zona Atlántica N-NO o el Convenio de Barcelona para la zona del Mediterráneo. Así, a través de los programas oficiales de vigilancia ambiental (***biomonitorización***) es posible determinar el estado ambiental del medio marino. La evaluación ambiental del medio marino, tradicionalmente se ha realizado mediante la identificación de los contaminantes químicos presentes en agua y sedimento, así como su acumulación en especies representativas (***bioindicadoras***). En este contexto, una de las especies más utilizadas como bioindicador de la contaminación ha sido el mejillón. El primer programa de vigilancia ambiental, también denominado programa "***Programa de Vigilancia del Mejillón***", se estableció en 1975 en Estados Unidos, usando el mejillón como especie indicativa de los niveles de ciertas clases de contaminantes en sus tejidos. El principal motivo por el que se propuso el mejillón como bioindicador es debido a su

carácter sésil y filtrador, actuando como un filtro natural de contaminación en una zona determinada. Además, su amplia distribución geográfica, gran capacidad de acumulación de contaminantes y fácil recolección y manipulación, los convirtió en una de las especies modelo para los programas de vigilancia ambiental y estudios toxicológicos.

En España, el Instituto Español de Oceanografía (IEO), y dentro de este, el Grupo de Contaminación Marina (Centros de Vigo y Murcia), lleva desarrollando los programas de vigilancia ambiental para la monitorización de la contaminación en el medio marino español desde hace más de 30 años, de forma coordinada, en los últimos años, con el Ministerio de Agricultura, Alimentación y Medio Ambiente (MAGRAMA). Estos programas, incluyen tanto el análisis de contaminantes en sedimento y biota, como los efectos biológicos que los contaminantes generan en los organismos, aplicando lo que se conoce como ***seguimiento integrado de la contaminación marina***.

En este contexto, para la identificación de los efectos biológicos de los organismos expuestos a contaminación, se utilizan baterías de ***biomarcadores.*** Los biomarcadores pueden definirse como medidas cuantitativas de los cambios a nivel celular, bioquímico, molecular o fisiológico, que pueden ser determinados a nivel de células, fluidos, tejidos u organismo, y que son indicativos de exposición o daño frente a la presencia de un contaminante.

Los biomarcadores son ampliamente utilizados en estudios de toxicología para identificar los efectos de la contaminación, sin embargo, su uso todavía no se ha implementado en los programas de vigilancia ambiental (ej: JAMP, MEDPOL), debido a su variabilidad en función de las condiciones ambientales, como son la disponibilidad de alimento, la temperatura o la salinidad. Esta variabilidad natural condiciona el metabolismo y la fisiología de los organismos bioindicadores, actuando como ***factores de confusión*** en la respuesta de los biomarcadores a la presencia de contaminantes. Por tanto, en el contexto de los programas de vigilancia ambiental integrados con mejillón, se han considerado como factores de confusión, todos aquellos factores, tanto exógenos (temperatura, disponibilidad de alimento, salinidad, tiempo de inmersión) como endógenos (ciclo reproductivo, edad) que pueden alterar, en mayor o menor medida, la respuesta de los biomarcadores a la contaminación.

Estudios previos llevados a cabo en los programas de vigilancia ambiental en la costa española por el grupo de Contaminación Marina del IEO, han evidenciado que las respuestas de los biomarcadores utilizados como indicadores de contaminación se ven muy afectadas por el ***estado de condición del mejillón*** lo que dificulta su uso como indicadores de la contaminación. En estos estudios, sólo se determinó el índice de condición como parámetro indicativo del estado de condición del mejillón. El índice de condición refleja tanto el estado nutritivo como el estado reproductor, los cuales están íntimamente ligados, por lo que, en el medio natural, resulta imposible aislar un efecto

del otro. Como consecuencia, los experimentos de laboratorio son de gran utilidad para la evaluación de los efectos biológicos provocados por los contaminantes bajo determinados escenarios ambientales. Por tanto, este tipo de estudios son indispensables para entender cómo el factor de confusión "*condición del mejillón*" está afectando la respuesta de los biomarcadores a la contaminación con la última finalidad de validar su uso en los programas de vigilancia ambiental.

HIPÓTESIS Y OBJETIVOS

La hipótesis de esta tesis fue que en los programas de vigilancia ambiental, el ***estado de condición del mejillón (estado nutritivo y reproductivo)*** determina los niveles de los biomarcadores, lo que enmascara su respuesta a la contaminación.

De acuerdo con esta hipótesis, el **objetivo general** de esta tesis consistió en determinar el modo en el que el estado de condición del mejillón está afectando a las respuestas de los biomarcadores a la contaminación.

Para ello se plantearon los siguientes objetivos específicos:

1. Identificar las variables biológicas que pueden actuar como ***factores de confusión*** en los programas de vigilancia ambiental. Se propuso establecer relaciones entre i) las variables biológicas y los biomarcadores y ii) los biomarcadores y la contaminación, considerando poblaciones naturales de mejillón utilizadas dentro de un programa de vigilancia ambiental.

2. Estudiar el efecto que el ***estado nutritivo del mejillón a nivel cuantitativo (Condición I***), tiene sobre la respuesta de los biomarcadores a la contaminación.

3. Estudiar el efecto que el ***estado nutritivo del mejillón a nivel cualitativo (Condición II***), tiene sobre la respuesta de los biomarcadores a la contaminación.

4. Estudiar el efecto que el ***estado reproductivo del mejillón (Condición III***), tiene sobre la respuesta de los biomarcadores a la contaminación.

Además, para un mejor conocimiento de las respuestas de los biomarcadores bajo los efectos de los factores de condición estudiados, en esta tesis se consideraron biomarcadores a distintos niveles de organización: respuestas fisiológicas (aclaramiento –CR-, eficiencia de absorción –AE- y respiración –RR- y la integración de todos ellos en el scope for growth –SFG-), inmunológicas (número total de hemocitos –THC-, viabilidad, fagocitosis y producción de especies reactivas de oxígeno por hemocitos -ROS-), respuestas celulares antioxidantes (superóxido dismutasa –SOD-, catalasa –CAT-, glutatión peroxidasa –GPx- y glutatión reductasa –GR-), respuestas celulares del

sistema II de la biotransformación (glutatión-S-transferasa –GST-) así como indicadores de daño celular como peroxidación lipídica de las membranas (LPO).

Esta tesis tiene un total de 4 capítulos, cada uno de los cuales recoge los resultados obtenidos tras el análisis y desarrollo de uno de los objetivos.

CAPÍTULO 1

En este capítulo, se llevó a cabo un estudio en profundidad de las variables biológicas del mejillón en el programa de vigilancia ambiental llevado a cabo en el año 2012 en la costa Atlántica N-NO, dentro del Programa de Seguimiento de la Contaminación Marina del IEO (SMP) (objetivo 1). Para ello, se recogieron medidas cualitativas y cuantitativas de las variables biológicas del mejillón: índices corporales, desarrollo gonadal, cuantificación de los componentes bioquímicos, entre otros, para identificar aquellas variables que pueden afectar la respuesta de los biomarcadores a la contaminación. Se consideró una batería de biomarcadores para identificar respuestas desde el nivel celular hasta el nivel de organismo: biomarcadores fisiológicos (SFG) y enzimáticos (CAT, GST, GR, LPO).

Como resultado de este estudio, se observó una gran variabilidad geográfica en las variables biológicas medidas en los mejillones, la cual, estuvo relacionada, principalmente, con la disponibilidad de alimento del sitio de muestreo. Se encontró una relación inversa entre la respuesta de enzimas antioxidantes y el estado nutritivo del mejillón. En cambio, se obtuvo una relación positiva entre la LPO y el estado nutritivo, y, por tanto, también con un estado metabólico más activo, el cual está asociado con una mayor producción de especies reactivas de oxígeno (ROS). También se encontró una relación inversa entre estado de condición del mejillón y las tasas de aclaramiento (CR) y, en consecuencia, con el SFG, sugiriendo que los mejillones mantienen una *memoria ecológica* del hábitat natural del que provienen. En general, no se detectó una relación directa entre la contaminación y los biomarcadores estudiados. Sin embargo, se observó una relación estadísticamente significativa entre los parámetros biológicos en el mejillón y los biomarcadores fisiológicos y bioquímicos estudiados. Como principal conclusión de este estudio se obtuvo que, cuando las variables ambientales son muy diferentes entre puntos de muestreo, como es el caso de las condiciones tróficas, el efecto que esas condiciones ambientales ejerce sobre los parámetros biológicos del mejillón puede enmascarar la respuesta de los biomarcadores a la contaminación.

CAPÍTULO 2

El principal objetivo de este estudio fue el de identificar el efecto que la ***disponibilidad de alimento*** y, consecuentemente, el ***estado nutritivo del mejillón***, tiene sobre la respuesta de los biomarcadores bajo condiciones controladas de laboratorio (objetivo 2). Por este motivo, mejillones muestreados de un punto de referencia fueron acondicionados a tres raciones distintas de alimento durante dos meses, para crear tres estados nutritivos bien diferenciados. Posteriormente, cada grupo de mejillones fue expuesto a dos concentraciones nominales de un tóxico modelo, fluoranteno (FLU), durante tres semanas. Se consideró una batería de biomarcadores para identificar respuestas desde nivel celular a nivel del organismo: biomarcadores fisiológicos (SFG) y enzimáticos (SOD, CAT, GR, GPx, GST y LPO).

Como resultado de este estudio se obtuvo que la mayoría de los biomarcadores estudiados (SFG, SOD, CAT y GPx) estuvieron fuertemente afectados por el estado nutritivo del mejillón, mostrando valores más altos en aquellos mejillones con peor estado nutritivo. Por el contrario, el efecto del tóxico no fue siempre evidente y, en general, se vio enmascarado por el efecto del estado nutritivo. Este estudio demostró que los contaminantes no son la única fuente capaz de modular la respuesta de los biomarcadores, y confirmó que el estado nutritivo del mejillón es un importante factor a tener en cuenta en el estudio de la repuesta de los biomarcadores a la contaminación puesto que puede alterar los valores de biomarcadores fisiológicos y bioquímicos.

CAPÍTULO 3

El principal objetivo de este estudio fue determinar el efecto que la ***calidad del alimento*** y, en consecuencia ***el estado nutritivo del mejillón***, tiene sobre la respuesta de algunos biomarcadores inmunológicos y bioquímicos usados como indicadores de contaminación. Para ello, mejillones procedentes de un punto de referencia fueron acondicionados durante seis semanas a dos tipos de dieta, una diatomea *Chaetoceros neogracile*, y un dinoflagelado *Heterocapsa triquetra*, seleccionados como dos tipos bien diferenciados de calidad de alimento. Estas dos especies difieren en muchos aspectos, principalmente en su biometría y su composición bioquímica. Tras el periodo de acondicionamiento, las dos condiciones de mejillón fueron expuestas a FLU durante una semana. Los biomarcadores bioquímicos estudiados fueron: SOD, CAT, GR, GST, LPO y los inmunológicos: número total de hemocitos, porcentaje de mortalidad de hemocitos, tasa y capacidad fagocítica, producción de ROS en hemocitos.

Los resultados mostraron diferencias en el comportamiento de asimilación de FLU en las dos condiciones. Aquellos mejillones alimentados con *C. neogracile* (C-mussels) mostraron mayores valores de FLU acumulado en sus tejidos que los alimentados con *H. triquetra* (H-mussels) y en un mayor impacto del tóxico sobre los biomarcadores estudiados. Este grupo de mejillones (C-mussels), mostró un aumento en la mortalidad de los hemocitos y una activación de los procesos de fagocitosis así como

de generación de especies reactivas de oxígeno por los hemocitos. Al mismo tiempo, algunos biomarcadores enzimáticos se vieron afectados (SOD, CAT y GR), presentando mayores actividades en presencia de FLU. Este estudio demostró que, no solo la exposición al tóxico, sino también la calidad del alimento, pueden afectar a la respuesta de los biomarcadores. En concreto, mejillones alimentados con *C. neogracile* mostraron mayor actividad de SOD, CAT y GR en los tratamientos control (sin tóxico). Además, en este estudio, para observar el efecto de la dieta bajo una misma concentración de tóxico, se utilizó un análisis de comparación de rectas de regresión (ANCOVA). El análisis ANCOVA evidenció que para una misma concentración de tóxico, el biomarcador GR respondió de forma diferente según la dieta con la que fueron alimentados los mejillones. Este hecho, sugirió que la calidad de la dieta puede ser considerada como un factor de confusión puesto que puede enmascarar la respuesta de los biomarcadores a la contaminación. En consecuencia, las condiciones tróficas del medio, incluyendo no sólo la cantidad, sino también la calidad del alimento, deben ser consideradas en los programas de vigilancia ambiental costera para una correcta interpretación de la respuesta de los biomarcadores a la contaminación.

CAPÍTULO 4

El programa de vigilancia ambiental desarrollado en el capítulo 1 de esta tesis, evidenció que existe una gran variabilidad en los parámetros biológicos de los mejillones analizados. En concreto, demostró que es posible encontrar mejillones en distintos estados reproductivos en un mismo muestreo. Puesto que ha sido descrito que la reproducción genera la activación de diversos procesos metabólicos en el mejillón y los biomarcadores están íntimamente ligados al metabolismo del animal, es necesario comprender el efecto que diferentes estados reproductivos ejercen sobre la respuesta de los biomarcadores a la contaminación. Por tanto, el objetivo de este capítulo (objetivo 4), fue el de estudiar el efecto que el ***estado reproductivo del mejillón*** tiene sobre los biomarcadores, bajo condiciones controladas de laboratorio (alimento y temperatura). Para llevar a cabo este estudio, se muestrearon mejillones de una zona de referencia en dos momentos del año, descritos como de reposo y de maduración sexual. Posteriormente, ambos grupos de mejillones fueron aclimatados a las condiciones de laboratorio durante una semana para evitar la posible influencia de factores externos. Tras la aclimatación, los mejillones fueron expuestos al tóxico modelo, FLU, durante un periodo de tres semanas. En este trabajo se desarrolló un profundo estudio histológico del mejillón para poder determinar diferencias en el estado reproductivo del mejillón e identificar posibles daños provocados por el tóxico. Igualmente, se utilizaron biomarcadores fisiológicos (SFG) y enzimáticos (SOD, CAT, GR, GPx y LPO) para observar posibles efectos del estado reproductivo.

Los resultados obtenidos mostraron que el efecto del estado reproductivo fue mayor que el del tóxico en la mayoría de los biomarcadores estudiados (SFG, SOD, CAT y GPx), siendo los valores de estos biomarcadores mayores durante el período de maduración sexual. Además, el desarrollo gonadal se vio afectado por la presencia del tóxico.. Durante el período de reposo sexual, se observó una mayor eficiencia de acumulación del tóxico, siendo el doble de la observada en época reproductiva. Esto provocó un efecto significativo del tóxico en algunos biomarcadores (CAT, GPx y SFG). Por el contrario, en la época reproductora se produjo un aumento en los niveles de los biomarcadores ligado a la gametogénesis, lo que enmascara el efecto del tóxico. Este estudio demostró que el estado reproductivo condiciona los niveles de los biomarcadores comúnmente usados en programas de vigilancia ambiental y que es capaz de modular el efecto de contaminación sobre los mismos. Esta información es esencial para la correcta interpretación de los biomarcadores en los programas de vigilancia ambiental donde se ha evidenciado que existen mejillones en diferentes épocas del estado reproductivo.

PRINCIPALES CONCLUSIONES

Respuesta de los biomarcadores en los programas de vigilancia ambiental

1. Se observó una ***gran variabilidad geográfica en los parámetros biológicos*** de las poblaciones naturales de mejillón muestreadas en el programa de vigilancia ambiental llevado a cabo en la costa Atlántica y Cantábrica española (SMP). El desarrollo gonadal y el contenido de reservas fueron los dos parámetros biológicos que mostraron una mayor variabilidad. Cuanto mayor fue el área de estudio considerada, mayor fue la variabilidad observada en las características biológicas, debido, principalmente, a las diferentes condiciones tróficas entre puntos de muestreo.

2. Se observaron dos patrones bien diferenciados en la respuesta de los biomarcadores a la *contaminación* y a las *variables biológicas* del mejillón. En la costa Atlántica no se apreció ninguna relación significativa entre la respuesta de biomarcadores y las variables biológicas. Por el contrario, en la costa Cantábrica si se observó un efecto de las variables biológicas en la respuesta de los biomarcadores estudiados, mientras que no se encontró una relación directa entre la respuesta de los biomarcadores y la contaminación. Este resultado puede explicarse gracias al efecto que los parámetros biológicos del mejillón tienen sobre los biomarcadores, los cuales pueden estar actuando como factores de confusión, modificando la respuesta de los biomarcadores a la contaminación.

3. Por tanto, los programas de vigilancia ambiental deberían considerar la medida de ***parámetros biológicos*** y ***ambientales***, para una correcta interpretación de los biomarcadores, siendo esta recomendación esencial en programas de vigilancia ambiental de gran extensión donde los organismos están expuestos a condiciones ambientales muy diversas.

Respuesta de los biomarcadores al estado de condición del mejillón

4. El estado nutritivo del mejillón**,** en términos de calidad del alimento, no tuvo ningún efecto significativo sobre los ***biomarcadores inmunológicos*** estudiados. En esta tesis, los biomarcadores inmunológicos no fueron considerados bajo otros tipos de condición, por tanto, no fue posible establecer la relación entre los biomarcadores inmunológicos y el estado de condición del mejillón.

5. El ***biomarcador SFG*** se vio fuertemente afectado por el estado nutritivo del mejillón. Este hecho, compromete el uso del SFG como biomarcador en los programas de vigilancia ambiental, puesto que estos programas están caracterizados por una gran variabilidad de las condiciones tróficas entre puntos y periodos de muestreo. Todos los parámetros fisiológicos que integran el SFG (***filtración, absorción*** y ***respiración***) se vieron fuertemente afectados por el estado nutritivo del mejillón, el cual fue directamente dependiente de su pasado nutritivo (disponibilidad de alimento en el medio) previo a la toma de medidas, a pesar de que estas medidas se tomaron bajo condiciones controladas de laboratorio. Por el contrario, el SFG no se vio afectado por el estado reproductivo.

6. Los ***biomarcadores bioquímicos*** medidos en la glándula digestiva del mejillón (***CAT, SOD, GPx*** *y* ***GR***) se vieron fuertemente afectados por el estado nutritivo del mejillón en sus dos vertientes (calidad y cantidad del alimento) pero, además, se vieron afectados por el estado reproductivo. Por ello, estos biomarcadores por si solos, no se deberían considerar como buenos indicadores de la contaminación en los programas de vigilancia ambiental. El biomarcador ***LPO*** mostró un comportamiento contrario a las enzimas antioxidantes. No se vio afectado por las variables experimentales estudiadas: estado nutritivo y reproductivo. El biomarcador ***GST***, por otra parte, no mostró ninguna relación clara con el estado de condición del mejillón.

Respuestas de los biomarcadores a la contaminación bajo diferentes estados de condición del mejillón

7. La ***bioacumulación de contaminantes*** dependió del estado de condición del mejillón. Por un lado, se observó una mayor acumulación en mejillones alimentados con mayor cantidad de alimento, en dietas ricas en lípidos y en aquellas dietas que favorecían la digestibilidad del alimento. Por otro lado, el estado reproductivo también fue un factor que influyó en la eficiencia de acumulación del contaminante, siendo mayor en aquellos mejillones que presentaron un estado de reposo sexual. Por tanto, la acumulación es un factor que depende, no solo de la concentración de contaminantes en el medio, sino también de la condición del mejillón.

8. Los ***biomarcadores inmunológicos*** se vieron afectados por la presencia del tóxico, aunque se observó una mayor respuesta inmune en los mejillones alimentados con *C. neogracile*. Por tanto la diferente respuesta de los parámetros inmunológicos observada en los mejillones dependiendo del tipo de dieta con la que fueron alimentados, sugiere que la calidad de la dieta actúa como un *factor de confusión*, modificando la respuesta de estos biomarcadores a la contaminación.

9. El efecto del estado nutritivo del mejillón sobre el ***biomarcador SFG*** fue mayor que el efecto de la contaminación. Esto se debe, principalmente, al fuerte efecto que las condiciones nutritivas del mejillón tuvieron en las tasas de filtración. Además, la respuesta del SFG a la contaminación fue dependiente del estado reproductivo siendo los mejillones en estado de reposo sexual, los únicos que mostraron un efecto negativo del SFG al tóxico. Considerando la gran variabilidad de los parámetros biológicos observados en los programas de vigilancia ambiental, es necesario realizar más estudios para validar el uso de este biomarcador como indicador de contaminación en este tipo de programas.

10. Las respuestas de los ***biomarcadores bioquímicos*** a la contaminación dependieron, en gran medida, del estado de condición del mejillón, en su doble vertiente, estado nutritivo y reproductivo. Los cambios biológicos observados en los experimentos de laboratorio, como son el estrés nutritivo o el estrés producido por la reproducción, elevaron los niveles de algunas actividades enzimáticas antioxidantes (***SOD, CAT, GPx*** y ***GR)***. Este aumento favoreció los procesos de desintoxicación, mostrando, por ejemplo, una menor bioaccumulacion de contaminantes. Como consecuencia, las enzimas antioxidantes, por sí solas, no pueden considerarse como un buen indicador de la contaminación. A pesar de que la **LPO** fue el único biomarcador bioquímico que no se vio afectado por el estado nutritivo del mejillón, este biomarcador tampoco se vio afectado por el contaminante usado en esta tesis (FLU). La ***GST***, por su parte, no mostró un patrón claro de respuesta ante la contaminación.

11. Los mejillones han sido clasificados como organismos resistentes a la contaminación. Por este motivo, muestran una amplia distribución geográfica ocupando incluso áreas muy contaminadas. Al mismo tiempo, son organismos intermareales, es decir, habitan en ambientes muy variables, lo QUE ha favorecido el desarrollo de complejos mecanismos adaptativos al entorno para cubrir sus necesidades energéticas. Estos procesos reguladores hacen que los mejillones sean extremadamente sensibles a la disponibilidad de alimento, siendo capaces de regular sus procesos biológicos para la captación de alimento a todos sus niveles, desde el nivel celular hasta el nivel fisiológico. Además, se trata de organismos oportunistas, por lo que son capaces de madurar reproductivamente si las condiciones ambientales son favorables. En esta tesis se ha evidenciado que los biomarcadores usados en los programas de vigilancia ambiental están estrechamente relacionados con los procesos biológicos del mejillón y como consecuencia, en condiciones naturales, la contaminación no es el único factor capaz de modular la respuesta de los biomarcadores. Por tanto, en los programas de vigilancia ambiental, es necesario considerar **el estado de condición del mejillón** para poder interpretar correctamente los datos obtenidos de la respuesta de los biomarcadores a la contaminación.

BIBLIOGRAPHY

BIBLIOGRAPHY

Aarab, N., Minier, C., Lemaire, S., Unruh, E., Hansen, P.D., Larsen, B.K., Andersen, O.K., Narbonne, J.F. 2004. Biochemical and histological responses in mussel (*Mytilus edulis*) exposed to North Sea oil and to a mixture of North Sea oil and alkylphenols. Mar.Environ. Res. 58, 437–441.

Aarab, N., Godal, B.F., Bechmann, R.K., 2011. Seasonal variation of histopathological and histochemical markers of PAH exposure in blue mussel (*Mytilus edulis L.*). Mar. Environ. Res. 71, 213–7. doi:10.1016/j.marenvres.2011.01.005

Abele, D., Brey, T., Philipp, E., 2009. Bivalve models of aging and the determination of molluscan lifespans. Exp. Geront. 44, 307-315.

Adams, S.M., 2005. Assessing cause and effect of multiple stressors on marine systems. Mar. Pollut. Bull. 51, 649–657

Alaee, M., Arias, P., Sjodin, A., Bergman, A. 2003. An overview of commercially used brtominated flame retardants, their applications, their use patterns in different countries/regions, and posible modes of release. Environ Int. 29, 683-689.

Albentosa, M., Labarta, U., Pérez-Camacho, A., Fernandez-Reiriz, M.J., Beiras, R., 1994. Fatty acid composition of Venerupis pullastra spat fed on different microalgae diets. Comp. Biochem. Physiol. Part A Physiol. 108, 639–648. doi:10.1016/0300-9629(94)90350-6

Albentosa, M., Labarta, U., Fernández-Reiriz, M.J., Pérez-Camacho, A., 1996. Fatty acid composition of *Ruditapes decussatus* spat fed on different microalgae diets. Comp. Biochem. Physiol. Part A Physiol. 113, 113–119. doi:10.1016/0300-9629(95)02041-1

Albentosa, M., Fernández-Reiriz, M.J., Labarta ,U., Pérez-Camacho, A., 2007. Response of two species of clams, *Ruditapes decussatus* and *Venerupis pullastra*, to starvation: Physiological and biochemical parameters. Comp. Biochem. Physiol. B. 146 (2), 241-249.

Albentosa, M., Viñas, L., Besada, V., Franco, M.A., González-Quijano, A., 2012a. First measurements of the scope for growth (SFG) in mussels from a large scale survey in the North-Atlantic Spanish coast. Sci. Tot. Environ. 435-436, 430-445.

Albentosa, M., Sánchez-Hernández, M., Campillo, J.A., Moyano, F., 2012b. Relationship between physiological measurements (SFG –scope for growth-) and the functionality of the digestive gland in *Mytilus galloprovincialis*. Comp. Biochem. Physiol. A. 163, 286-295.

Ackerman, J., Nishizaki, M. 2004. The effect of velocity on the suspension feeding and growth of the marine mussels *Mytilus trossulus* and *M. californianus*: implications for niche separation. J Marine Syst 49(1-4), 195- 207.

Anderson, T., Forlin, L., Hardig, J., Larsson, A. 1988. Physiological disturbances in fish living in coastal water polluted with leached kraft mill effluents. C. J. Fish. Aquat. Sci. 45, 1525-1536

Anderlini, V.C., 1992. The effect of sewage on trace metal concentrations and scope for growth in *Mytilus edulis aoteanus* and *Perna canaliculus* from Wellington Harbour, New Zealand. Sci. Total Environ. 125, 263–287. doi:10.1016/0048-9697(92)90396-A

Ansari, T.M., Marr, I.L, Tariq, N. 2004. Heavy metals in marine pollution perspective-a mini review. J. Appl. Sci. 4(1), 1-20.

Alunno-Bruscia, M., Bourget, E., Frechette, M., 2001. Shell allometry and length-mass-density relationship for *Mytilus edulis* in an experimental food-regulated situation. Mar. Ecol. Prog. Ser. 219, 177-188.

Al-Subiai, S.N., Arlt, V.M., Frickers, P.E., Readman, J.W., Stolpe, B., Lead, J.R., Moody, A.J., Jha, A.N., 2012. Merging nano-genotoxicology with eco-genotoxicology: an integrated approach to determine interactive genotoxic and sub-lethal toxic effects of C(60) fullerenes and fluoranthene in marine mussels, *Mytilus sp*. Mutat. Res. 745, 92–103. doi:10.1016/j.mrgentox.2011.12.019

Álvarez, I., Gómez-Gesteira, M., De Castro, M., Lorenzo, M.N., Crespo, A.J.C., Días, J.M., 2011. Comparative analysis of upwelling influence between the western and northern coast of the Iberian Peninsula. Cont. Shelf. Res. 31, 388-399.

Angilletta, M.J., Sears, M.W., 2000. The metabolic cost of reproduction in an oviparous lizard. Funct. Ecol. 14, 39–45.

Ansaldo, M., Sacristán, H., Wider, E., 2007. Does starvation influence the antioxidant status of the digestive gland of *Nacella concinna* in experimental conditions?. Comp. Biochem. Physiol. C. 146, 118-123.

Ansell, A.D., 1973. Oxygen consumption by the bivalve *Donax vittatus* (da Costa). J. Exp. Mar. Biol. Ecol. 11, 311–328.

Auffret M and Oubella R. 1994. Cytometric parameters of Bivalve Molluscs: Effect of Environmental factors, in: Stolen, J.S., Fletcher, T.C. (Eds.), Modulators of fish immune responses, Vol I. Models for environmental toxicantology biomarkers immunomodulators. SOS Publications, Fair Haven, NJ 07704-3303, USA

Auffret, M., Mujdzic, N., Corporeau, C., & Moraga, D. 2002. Xenobiotic-induced immunomodulation in the European flat oyster, *Ostrea edulis*. Mar. environ.res. 54(3), 585-589.

Babarro, J.M.F., Fernández-Reiriz, M.J., Labarta, U., 2000. Feeding behaviour of seed mussel *Mytilus galloprovincialis*: Environmental parameters and seed origin. J. Shellfish. Res. 19(1), 195-201.

Balbi, T., Smerilli, A., Fabbri, R., Ciacci, C., Montagna, M., Grasselli, E., Brunelli, A., Pojana, G., Marcomini, A., Gallo, G., Canesi, L. 2014. Co-exposure to n-TiO 2 and Cd2+ results in interactive effects on biomarker responses but not in increased toxicity in the marine bivalve *M. galloprovincialis.* Sci. Total. Environ. 493, 355-364.

Barber, B.J., Blake, N.J., 1991. Reproductive physiology. In: Shumway, SE, editor. Scallop: biology, ecology and aquaculture. Amsterdam: Elsevier, 377-428.

Barceló, D., 2003. Emerging pollutants in water analysis. TRAC Trends in Analytical Chemistry, 22, 14-16.

Barni, S., Gonzalez, M.F., Miglioranza, K.S.B., 2014. Assessment of persistent organic pollutants accumulation and lipid peroxidation in two reproductive stages of wild silverside (*Odontesthes bonariensis*). Ecotoxicol. Environ. Saf. 99, 45–53. doi:10.1016/j.ecoenv.2013.10.012

Baumard P, Budzinski H, Garrigues P, Sorbe JC, Burgeot T, Bellocq J. Concentrations of PAHs (polycyclic aromatic hydrocarbons) in various marine organisms in relation to those in sediments and to trophic level. Mar Pollut Bull 1998; 36: 951–60.

Baumard, P., Budzinski, H., Garrigues, P., 1998. Polycyclic aromatic hydrocarbons in sediments and mussels of the western Mediterranean Sea. Environ. Toxicol. Chem. 17, 765-776

Baussant, T., Ortiz-Zarragoitia, M., Cajaraville, M.P., Bechmann, R.K., Taban, I.C., Sanni, S., 2011. Effects of chronic exposure to dispersed oil on selected reproductive processes in adult blue mussels (*Mytilus edulis*) and the consequences for the early life stages of their larvae. Mar. Pollut. Bull. 62, 1437–45. doi:10.1016/j.marpolbul.2011.04.029

Bayne, B.L., 1973. Physiological changes in *Mytilus edulis* L. induced by temperature and nutritive stress. J. Mar. Biol. Assoc. UK. 53, 39–58.

Bayne, B. L., 1976. Aspects of reproduction in bivalve molluscs. In: Wiley M, editor. Estuarine Processes, Vol I. New York, Academic Press, 432-448.

Bayne, B.L., 1976. Estuarine Processes, Estuarine Processes. Elsevier. doi:10.1016/B978-0-12-751801-5.50043-5

Bayne, B.L., Widdows, J., 1978. The physiological ecology of two populations of *Mytilus edulis* L . Oecologia 37, 137-162 .

Bayne, B.L., 1984. Aspects of reproductive behaviour within species of bivalve molluscs. In: Wolf Engels, Wallis H. Clark (Eds.), Advances in Invertebrate Reproduction, vol.3. Amsterdam, NewYork, pp.357–366.

Bayne, B. L. 1998. The physiology of suspension feeding by bivalve mol- luscs: an introduction to the Plymouth "TROPHEE" workshop. J. Exp. Mar. Biol. Ecol. 219, 1-19.

Bayne, B.L., Newell, R.C., 1983. Physiological energetics of marine molluscs. In: Wilbur KM, Saleuddin AS, editors. The Mollusca. New York, Academic Press, 407–515.

Bayne, B.L., 1993. Feeding physiology of bivalves: time-dependence and compensation for changes in food availability. In: Dame RF, editor. Bivalve Filter Feeders in Estuarine and Coastal Ecosystem Processes. Berlin: Springer-Verlag, 1–24.

Bayne, B.L., 1998. The physiology of suspension feeding by bivalve molluscs: an introduction to the Plymouth "TROPHEE" workshop. J. Exp. Mar. Biol. Ecol. 219, 1-19.

Bayne, B. L., Thompson, R. J. 1970. Some physiological consequences of keeping *Mytilus edulis* in the laboratory. Helgola'nder Wissenschaftliche Meeresuntersuchungen, Hegloland, 20, 526-52

Bayne, B.L., Holland, D.L., Moore, M.N., Lowe, D.M., Widdows, J., 1978. Further studies on the effects of stress in the adult on the eggs of *Mytilus edulis*. J mar boil Ass UK 58, 825-841.

Bayne, B.L., Widdows, J., Moore, M.N., Salkeld, P., Worrall, C.M., Donkin, P., 1982. Some ecological consequences of the physiological and biochemical effects of petroleum compounds on marine molluscs. Philos. Trans. R. Soc. Lond., B 297, 219–239.

Bayne, B.L., Newell, R.C., 1983. The Mollusca, The Mollusca. Elsevier. doi:10.1016/B978-0-12-751404-8.50017-7

Bayne, B.L., Widdows, J., 1978. The physiological ecology of two populations of *Mytilus edulis L*. Oecologia 37, 137–162. doi:10.1007/BF00344987

Bayne, B.L.,Gabbot, P.A., Widdows, J. 1975. Some effects of stressin the adult on the eggs and lavae of *Mytilus edulis L*. J. Exp. Mar. Biol. Ecol. 1:220-235.

Bayer M, Hellio C, Maréchal JP, Frank W, Lin W, Weber H, Proksch P. 2011. Antifouling bastadin congeners target mussel phenoloxidase and complex copper(II) ions. Mar Biotechnol (NY). 13(6):1148-58. doi: 10.1007/s10126-011-9378-3.

Béguel, J.-P., Huvet, A., Quillien, V., Lambert, C., Fabioux, C., 2013. Study of the antioxidant capacity in gills of the Pacific oyster *Crassostrea gigas* in link with its reproductive investment. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 157, 63–71. doi:10.1016/j.cbpc.2012.10.004

Beiras, R., Camacho, a. P., Albentosa, M., 1994. Comparison of the scope for growth with the growth performance of *Ostrea edulis* seed reared at different food concentrations in an open-flow system. Mar. Biol. doi:10.1007/BF00349561

Beiras, R., Durán, I., Parra, S., Urrutia, M.B., Besada, V., Bellas, J., Viñas, L., Sánchez-Marín, P., González-Quijano, A., Franco, M.A., Nieto, O., González, J.J., 2012. Linking chemical contamination to biological effects in coastal pollution monitoring. Ecotoxicol. 21, 9-17.

Beliaeff, B., Burgeot, T. 2002. Integrated biomarker response: a useful tool for ecological risk assessment. Environ. Toxicol. Chem. 21, 1316– 1322.

Bellas, J., Nieto, O., Beiras, R., 2011. Integrative assessment of coastal pollution: Development and evaluation of sediment quality criteria from chemical contamination and ecotoxicological data. Cont. Shelf. Res. 31, 448-456.

Bellas, J., 2014. The implementation of the Marine Strategy Framework Directive: Shortcomings and limitations from the Spanish point of view. Mar. Policy 50, 10–17. doi:10.1016/j.marpol.2014.05.004

Bellas, J., Albentosa, M., Vidal-Liñán, L., Besada, V., Franco, M.A., Fumega, J., González-Quijano, A., Viñas, L., Beiras, R., 2014. Combined use of chemical, biochemical and physiological variables in mussels for the assessment of marine pollution along the N-NW Spanish coast. Mar. Environ. Res. 96, 105-117.

Ben Othman, H., Leboulanger, C., Le Floc'h, E., Mabrouk, H.H., Hlaili, A.S., 2012. Toxicity of benz(a)anthracene and fluoranthene to marine phytoplankton in culture: does cell size really matter? J. Hazard. Mater. 243, 204–11. doi:10.1016/j.jhazmat.2012.10.020

Bergamini, E., Cavallini, G., Donati, A., Gori, Z., 2003. The ant-ageing effects of caloric restriction may involve stimulation of macroautophagy and lysosomal degradation, and can be intensified pharmacologically. Biomed. Pharmacother. 57, 203–208.

Bernier, J., Brousseau, P., Krzystyniak, K., Tryphonas, H., Fournier, M. 1995. Immunotoxicity of heavy metals in relation to Great Lakes. Environ Health Perspect, 103: 23–24

Besada, V., Andrade, J.M., Schultze F., González, J.J., 2011. Comparison of the 2000 and 2005 spatial distributions of heavy metals in wild mussels from the Nort-Atlantic Spanish coast. Ecotoxicol. Environ. Saf. 74, 373-381.

Besada, V., Sericano, J.L., Schultze, F., 2014. An assessment of two decades of trace metals monitoring in wild mussels from the Northwest Atlantic and Cantabrian coastal areas of Spain, 1991-2011. Environ. Inter. 71, 1-12.

Blandin, P. 1986. Bioindicateurs et diagnostic des systèmes écologiques. *Bulletin d'écologie,* 17(4), 211-307.

Bligh, E.G., Dyer, W.J., 1959. A rapid method for total lipid extraction and purification. J. Biochem. Physiol. 37, 911-917.

Bocchetti, R., Regoli, F., 2006. Seasonal variability of oxidative biomarkers, lysosomal parameters, metallothioneins and peroxisomal enzymes in the Mediterranean mussel *Mytilus galloprovincialis* from Adriatic Sea. Chemosphere. 65, 913-921.

Bode, A., Casas, B., Fernández, E., Marañón, E., Serret, P., Varela, M., 1996. Phytoplankton biomass and production in shelf waters off NW Spain: spatial and seasonal variability in relation to upwelling. Hydrobiologia. 341, 225-234.

Bode, A., Varela, M., Prego, R., 2011. Continental and marine resources of organic matter and nitrogen for rias of northern Galicia (Spain). Mar. Ecol. Prog. Ser. 437, 13-26.

Bodin, N., Burgeot, T., Stanisière, J.Y., Bocquené, G., Menard, D., Minier, C., Boutet, I., Amat, a, Cherel, Y., Budzinski, H., 2004. Seasonal variations of a battery of biomarkers and physiological indices for the mussel *Mytilus galloprovincialis* transplanted into the northwest Mediterranean Sea. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 138, 411–27. doi:10.1016/j.cca.2004.04.009

Boening, D. W. 1999. An evaluation of bivalves as biomonitors of heavy metals pollution in marine waters. Environ. Monitor. Assess. *55*(3), 459-470.

Borkovic, S.S., Saponjic, S.J., Pavlovic, Z.S., Blagojevic, P.D., Milosevic, M.S., Kovacevic, B.T., Radojicic, M.R., Spasic, B.M., Zikic, V.R., Saicic, S.Z., 2005. The activity of antioxidant defence enzymes in the mussel *Mytilus galloprovincialis* from the Adriatic Sea. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 141, 366-374.

Borkovic-Mitic, S., Pavlovic, S., Perendija, B., Despotovic, S., Gavric, J., Gacic, Z., Saicic, Z., 2013. Influence of some metal concentrations on the activity of antioxidant enzymes and concentrationsof vitamin E and SH-groups in the digestive gland and gills of the freshwater bivalve *Unio tumidus* from the Serbian part of Sava River. Ecological Indicators. 32, 212-221.

Botas J., Fernandez E., Bode, A., Anadon, R., 1990. A persistent upwelling off theCentral Cantabrian Coast (Bay of Biscay). Estuarine Coastal Shelf. Sci. 30, 185-199.

Bougrier, S., Hawkins, A.J.S., Héral, M. 1997. Preingestive selection of different microalgal mixtures in *Crassotrea gigas* and *Mytilus edulis*, analysed by flow cytometry. Aquaculture. 150, 123-134.

Bourgeault, a, Gourlay-Francé, C., 2013. Monitoring PAH contamination in water: comparison of biological and physico-chemical tools. Sci. Total Environ. 454-455, 328–36. doi:10.1016/j.scitotenv.2013.03.021

Bouilly, K., Gagnaire, B., Bonnard, M., Thomas-Guyon, H., Renault, T., Miramand, P., Lapègue, S., 2006. Effects of cadmium on aneuploidy and hemocyte parameters in the Pacific oyster, *Crassostrea gigas*. Aquat. Toxicol. 78, 149–56. doi:10.1016/j.aquatox.2006.02.028

Bouzas A, Aguado D, Martí N, Pastor J, Herráez R, Campins P, et al. Alkylphenols and polycyclic aromatic hydrocarbons in eastern Mediterranean Spanish coastal marine bivalves. Environ Monit Assess 2011;176:169–81.

Box, A., Sureda, A., Galgani, F., Pons, A., Deudero, S., 2007. Assessment of environmental pollution at Balearic Islands applying oxidative stress biomarkers in the mussel *Mytilus galloprovincialis.* Comp. Bio. Physiol. Part C: Toxicol Pharmacol. 146 (4), 531-539.

Brillant, M.G.S., MacDonald, B.A., 2002. Postingestive selection in the sea scallop (*Placopecten magellanicus*) on the basis of chemical properties of particles. Mar. Biol. 141(3), 457–65.

Brown, M.R., Jeffrey, S.W., Volkman, J.K., Dunstan, G.., 1997. Nutritional properties of microaglae for mariculture. Aquaculture. 151, 315–331.

Brunk, U., Cadenas, E. 1988. The potential intermédiate role of lysosomes in oxygen free radical pathology. APMIS. 96, 3-13.

Budge, S.M., Parrish, C.C., Mckenzie, C.H., 2001. Fatty acid composition of phytoplankton, settling particulate matter and sediments at a sheltered bivalve aquaculture site. Mar. Chem. 76 (4), 285-303.

Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. In: Fleicher P, Parker L , editors. Methods in Enzymology, vol 52. Academic Press, New York, 302-310.

Cabanas, J.M., 2000. Variabilidad temporal en las condiciones oceanográficas de las aguas de la plataforma continental gallega. Algunas consecuencias biológicas, PhD

Thesis, Facultad de Ciencias. Departamento de Ecología y Biología Animal. Universidad de Vigo, 1-155.

Caceres-Martinez, J., Figueras, A., 1998. Long-term survey on wild and cultured mussels (*Mytilus galloprovincialis* Lmk) reproductive cycles in the Ria de Vigo (NW Spain). Aquacualture.162, 141-156.

Caceres-Martinez, J., Figueras, A., 2007. Estudios sobre el ciclo gonadal. In: Figueras A, editor. Biología y Cultivo del Mejillón (*Mytilus galloprovincialis*) en Galicia. Madrid: Consejo Superior de Investigaciones Científicas, 47-60.

Cajaraville, M.P., Marigómez, J.A., Angulo, E. 1991. Automated measurement of lysosomal structure alterations in oocytes of mussels exposed to petroleum hydrocarbons. Arch. Environ. Contam. Toxicol. 21, 395-400.

Cajaraville, M.P., Marigómez, J.A., Angulo, E., 1992. Comparative effects of the water accommodated fraction of three oils on mussels. 1. Survival, growth and gonad development. Comp. Biochem. Physiol., 102C (1), 103-112.

Cajaraville, M.P., Bebianno, M.J., Blasco , J., Porte, C., Sarasquete, C., Viarengo, A., 2000. The use of biomarkers to assess the impact of pollution in coastal environmental on the Iberian Peninsula: a practical approach. Sci. Total. Environ. 247, 295–311.

Campillo, J.A., Albentosa, M., Valdés, N.J., Moreno-González, R., León, V.M., 2013. Impact assessment of agricultural inputs into a Mediterranean coastal lagoon (Mar Menor, SE Spain) on transplanted clams (*Ruditapes decussatus*) by biochemical and physiological responses. Aquat. Toxicol. 142-143, 365-379.

Canesi, L., Viarengo, A., 1997. Age-related differences in glutathione metabolism in mussel tissues (Mytilus edulis L.). Comp. Biochem. Physiol. B. 116, 217-221.

Canty, J.A. Hagger, R.T. Moore, L. Cooper, T.S. Galloway. 2007. Sublethal impact of short term exposure to the organophosphate pesticide azamethiphos in the marine mollusc *Mytilus edulis*, Mar. Pollut. Bull. 54 (4), 396-402

Carballa, M., Omil, F., Lema, J.M., Llompart, M., García-Jares, C., Rodríguez, I., Gómez, M., Ternes, T., 2004. Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. Water Res. 38, 2918–2926. doi:10.1016/j.watres.2004.03.029

Cazenave, J., Bistoni, M.A., Pesce, S.F., Wunderlin, D.A., 2006. Differential detoxification and antioxidant response in diverse organs of *Corydoras paleatus* experimentally exposed to microcystin RR. Aquat. Toxicantol. 76, 1–12.

Cermeño, P., Marañón, E., Pérez, V., Serret, P., Fernández, E., Castro, C.G., 2006. Phytoplancton size structure and primary production in a highly dynamic coastal

ecosystem (Ría de Vigo, NW-Spain): Seasonal and short-time scale variability. Estuar. Coast. Shelf Sci. 67, 251-266.

Chandurvelan, R., Marsden, I.D., Gaw, S., Glover, C.N., 2013. Field-to-laboratory transport protocol impacts subsequent physiological biomarker response in the marine mussel, *Perna canaliculus*. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 164, 84–90. doi:10.1016/j.cbpa.2012.10.011

Chandurvelan, R., Marsden, I.D., Gaw, S., Glover, C.N., 2012. Impairment of green-lipped mussel (*Perna canaliculus*) physiology by waterborne cadmium: Relationship to tissue bioaccumulation and effect of exposure duration. Aquat. Toxicol. 124-125, 114–124. doi:10.1016/j.aquatox.2012.07.013

Chauvaud, L., Donval, A., Thouzeau, G., Paulet, Y.-M., Nézan, E., 2001. Variations in food intake of *Pecten maximus (L.)* from the Bay of Brest (France): Influence of environmental factors and phytoplankton species composition. Comptes Rendus l'Académie des Sci. - Ser. III - Sci. la Vie 324, 743-755. doi:10.1016/S0764-4469(01)01349-X

Cheng, T.C. 1981 Bivalves, in: Ratcliffe, N.A., Rowley, A.F. (Eds.). Invertebrate blood cells, Vol II. Academic press, London, pp: 321-300.

Cheung, C.C.C., Zheng, G.J., Li, A.M.Y., Richardson, B.J., Lam, P.K.S., 2001. Relationships between tissue concentrations of polycyclic aromatic hydrocarbons and antioxidative responses of marine mussels, *Perna viridis* 52, 189–203.

Cho, S.-M., Jeong, W.-G., 2005. Spawning impact on lysosomal stability of the Pacific Oyster, *Crassostrea gigas*. Aquaculture 244, 383–387. doi:10.1016/j.aquaculture.2004.12.013

Claiborne, A., 1985. Catalase activity. In: Greenwald, R.A. , editor. Handbook of Methods for Ocygen. Radical Reserarch. CRC Press, Boca Raton, FL, 283-284.

Clark, R.B. 2001. Metals. In: Oxford University press (Eds). Marine Pollution, Fifth Edition. Oxford University Press Inc. New York. pp236.

Clarke, K.R., Gorley, R.N. 2006. PRIMER v6: user manual/tutorial. PRIMER-E, Plymouth

Clements, W.H., 2000. Integrating effects of contaminants across levels of biological organization: An overview. J. Aquat. Ecosyst. Stress Recover. 7, 113–116. doi:10.1023/A:1009927612391

Coles, J.A., Farley, S.R., Pipe, R.K., 1994. Effects of fluoranthene on the immunocompetence of the common marine mussel, *Mytilus edulis*. Aquat. Toxicol. 30, 367–379. doi:10.1016/0166-445X(94)00051-4

Conover, R.J., 1966. Assimilation of organic matter by zooplankton. Limnol. Oceanogr. 11, 338–45.

Costas, M., Prego, R., Filgueiras, A.V., Bendicho, C., 2011. Land-ocean contribution of arsenic through a river-estuary-ria system (SW Europe) under the influence of arsenopyrite deposit in the fluvial basin. Sci. Total. Environ. 412-413, 304-314.

Coulaud, R., Geffard, O., Xuereb, B., Lacaze, E., Quéau, H., Garric, J., Charles, S., Chaumot, A., 2011. In situ feeding assay with *Gammarus fossarum* (Crustacea): Modelling the influence of confounding factors to improve water quality biomonitoring. Water Research. 45, 6417-6429.

Cózar, A., Echevarría, F., González-Gordillo, J.I., Irigoien, X., Ubeda, B., Hernández-León, S., Palma, A.T., Navarro, S., García-de-Lomas, J., Ruiz, A., Fernández-de-Puelles, M.L., Duarte, C.M., 2014. Plastic debris in the open ocean. Proc. Natl. Acad. Sci. U. S. A. 111, 10239– 10244. doi:10.1073/pnas.1314705111

Cranford, P.J., Emerson, C.W., Hargrave, B.T., Milligan, T.G., 1998. In situ feeding and absorption responses of sea scallops *Placopecten magellanicus* (Gmelin) to storm - induced changes in the quantity and composition of the seston. J. Exp. Mar. Biol. Ecol. 219, 5-70.

Cranford, P.J., Hill, P.S., 1999. Seasonal variation in food utilization by the suspension – feeding bivlave molluscs *Mytilus edulis* and *Placopecten magellanicus*. Mar. Ecol. Prog. Ser. 190, 223- 239.

Crandford, P.J., Ward, J.E., Shumway, S.E. 2011. Bivalve filter feeding: variability and limits of the aquaculture biofilter. In: Shumway SE, editor. Shellfish Aquaculture and the Environment. John Wiley & Sons, 81-124.

Crosby, M.P., Gale, L.D., 1990. A review and evaluation of bivalve condition index methodologies with a suggested standard method. J. Shellfish Res. 9, 233–237.

Dachs, J., Mejanelle, L., 2010. Organic pollutants in coastal waters, sediments, and biota: A relevant driver for ecosystems during the anthropocene?. Estuaries and Coasts 33, 1-14.

Dahlhoff, E.P., Stillman, J.H., Menge, B.A., 2002. Physiological community ecology: variation in metabolic activity of ecologically important rocky intertidal invertebrates along environmental gradients. Integr. Comp. Biol. 42, 862–871.

Dalsgaard, J., St John, M., Jattner, G., Müller-Navarra, D., Hagen, W., 2003. Fatty acid trophic markers in the pelagicmarine food environment. Adv. Mar. Biol. 46, 226–340.

Davies, I.M., Vethaak, D., 2012. ICES cooperative research report recherché collectives no. 315 november 2012 Integrated marine environmental monitoring of chemicals and their effects Editors.

Devin, S., Burgeot, T., Giambérini, L., Minguez, L., Pain-Devin, S. 2014. The Integrated Biomarker Response revisited: optimization to avoid misuse. Environ. Sc. Pollu. Res . 21, 2448-2454.

De Boer, J., 1988. Chlorobiphenyls in bound and non-bound lipids of fishes; comparison of different extraction methods. Chemosphere. 17,1803-1810.

De Castro, M., Gomez-Gesteira, M., Alvarez, I., Cabanas, J.M., Prego, R., 2008. Characterization of fall-winter upwelling recurrence along the Galicianwestern coast (NW Spain) from 2000 to 2005: dependence on atmosphericforcing. J. Mar. Systems. 72, 145–148.

De Luca-Abbott, S. B., Richardson, B. J., McClellan, K. E., Zheng, G. J., Martin, M., Lam, P. K. 2005. Field validation of antioxidant enzyme biomarkers in mussels *(Perna viridis)* and clams (*Ruditapes philippinarum*) transplanted in Hong Kong coastal waters. Mar. Poll. Bull., 51(8), 694-707.

Defossez, J. M., Hawkins, A.J.S. 1997. Selective feeding in shellfish: size–dependent rejection of large paticles within pseudofaeces from *Mytilus edulis*, *Ruditapes philippinarum* and *Tapes decussatus.* Mar. Biol. 129,139- 147.

Delaporte, M., Soudant, P., Moal, J., Kraffe, E., Marty, Y., Samain, J.-F., 2005. Incorporation and modification of dietary fatty acids in gill polar lipids by two bivalve species Crassostrea gigas and Ruditapes philippinarum. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 140, 460–70. doi:10.1016/j.cbpb.2005.02.009

Depledge, M.H., 2009. Novel approaches and technologies in pollution assessment and monitoring: a UK perspective. Ocean Coast. Manag. 52, 336–434

De Wit, C.A. 2002. An overview of brominated flame retardants in the environment. *Chemosphere*. 46, 583-624.

De Zwaan, A., Zandee, D.I., 1972. Body distribution and seasonal changes in the glycogen content of the common sea mussel Mytilus edulis. Comp. Biochem. Physiol. A. 43, 53-58.

Di Giulio, R.T., Washburn, P.C., Wenning, R.J., Winston, G.W., Jewell, C.S., 1989. Biochemical responses in aquatic animals: a review of determinants of oxidative stress. Environ. Toxicol. Chem. 8, 1103–1123.

Dissanayake, A., Galloway, T.S., Jones, M.B., 2008. Nutritional status of *Carcinus maenas* (Crustacea: Decapoda) influences susceptibility to contaminant exposure. Aquat. Toxicol. 89, 40–46. doi:10.1016/j.aquatox.2008.05.014

Donaghy, L., Lambert, C., Choi, K. S., Soudant, P. 2009. Hemocytes of the carpet shell clam *(Ruditapes decussatus*) and the Manila clam (*Ruditapes philippinarum*): current knowledge and future prospects. Aquaculture, 297(1), 10-24.

Donkin, P., Widdows, J., Evans, S. V., Worrall, C. M. & Carr, M., 1989. Quantitative structure-activity relationships for the effect of hydrophobic organic chemicals on rate of feeding by mussels (*Mytilus edulis*). Aquatic Toxicology, 14, 277-294.

Donkin, P., Widdows, J., Evans, S.E., Worrall, C.M., Carr, M. 1990. Quantitative structure-activity relationships for the effect of hydrophobic chemicals on rate of feeding by mussels (*Mytilus edulis).* Aquat. Toxicol. 14, 277–294.

Dowd, W.W., Felton, C. A., Heymann, H.M., Kost, L.E., Somero, G.N. 2013. Food availability, more than body temperature, drives correlated shifts in ATP-generating and antioxidant enzyme capacities in a population of intertidal mussels (*Mytilus californianus*). J. Exp. Mar. Bio. Ecol. 449, 171–185. doi:10.1016/j.jembe.2013.09.020

Dubois, M., Gilles, K., Hamilton, J., Rebers, P., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28(3), 350–356.

Dyrynda, E.A., Law, R.J., Dyrynda, P.E.J., Kelly, C.A., Pipe, R.K., Ratcliffe, N.A., 2000. Changes in inmune parameters of natural mussel *Mytilus edulis* populations following a major oil spill ('Sea Empress', Wales, UK). Mar Ecol Prog Ser 206: 155-170.

Eertman R.H.M., Groenink C.L.F.M.G., Sandee B, Hummel H., 1995. Responses of the Blue Mussel *Mytilus edulis L*. Following Exposure to PAHs or Contaminated sediment. Mar Env Res, 39: 169-173.

Ellis, R.P., Parry, H., Spicer, J.I., Hutchinson, T.H., Pipe, R.K., Widdicombe, S., 2011. Immunological function in marine invertebrates: responses to environmental perturbation. Fish. Shell. Immunol. 30, 1209–22. doi:10.1016/j.fsi.2011.03.017

European Commission. Directive 2008/56/EC of the European Parliament and of the Council of 17 June 2008, establishing a framework for community action in the field of marine environmental policy (Marine Strategy Framework Directive). 2008. Off. J. Eur. Union. L164, 19–40.

Faria, M., Ochoa, V., Blázquez, M., Juan, M.F.S., Lazzara, R., Lacorte, S., Soares, A.M.V.M., Barata, C., 2014. Separating natural from anthropogenic causes of

impairment in Zebra mussel (*Dreissena polymorpha*) populations living across a pollution gradient. Aquat. Toxicol. 152, 82–95. doi:10.1016/j.aquatox.2014.03.020

Farmaindustria, 2010. Memoria anual 2010

Farrar, D., Alexander, L.C., Yuan, L.L., Gerritsen, J. 2015. Regional observational studies: Adressing confounding. In: Norton, S.B., Cormier, S.M., Suter, G.W (Eds). Ecological causal assessment. CRC Press, pp. 2015 - 513

Feist, S.W., Bignell, J., Stentiford, G.D., 2006. Histological changes in caged mussels (*Mytilus sp*.) and cod (*Gadus morhua)* at contaminant gradients in the German Bight and the Statfjord offshore oil industry area in the North Sea. In: Hyllan, K., Lang, T., Vethaak, D. (Eds.), Biological Effects of Contaminants in Marine Pelagic Ecosystems. SETAC Press, Brussels, Belgium, pp. 311–327

Fernández, B., Campillo, J.A., Martínez-Gómez, C., Benedicto, J., 2010a. Antioxidant responses in gills of mussel (*Mytilus galloprovincialis*) as biomarkers of environmental stress along the Spanish Mediterranean coast. Aquat. Toxicol. 99, 186-197.

Fernández, B., Albentosa, M., Viñas, L., Franco, A., González, J.J., Campillo, J.A., 2010b. Integrated assessment of water quality of the Costa da Morte (Galicia, NW Spain) by means of mussel chemical, biochemical and physiological parameters. Ecotoxicology. 19, 735-750.

Fernández, B., Campillo, J.A., Martínez-Gómez, C., Benedicto, J., 2012. Assessment of the mechanisms of detoxification of chemical compounds and antioxidant enzymes in the digestive gland of mussels, *Mytilus galloprovincialis*, from Mediterranean coastal sites. Chemosphere. 87, 1235-1245.

Fernández-Reiriz, M.J., Navarro, J.M., Cisternas, B.A., Babarro, J.M.F., Labarta, U., 2013. Enzymatic digestive activity and absorption efficiency in *Tagelus dombeii* upon Alexandrium catenella exposure. Helgol Mar Res 67, 653-661.

Figueiras, R. Labarta, U., Fernández-Reiriz, M.J. 2002. Coastal upwelling, primary production and mussel growth in the Rías Baixas of Galicia. Hydrobiologia. 484, 121-131

Filgueira, R., Labarta, U., Fernández-Reiriz, M.J., 2008. Effect of condition index on allometric relationships of clearance rate in *Mytilus galloprovincialis* Lamarck, 1819. Rev. Biol. Mar. Oceanogr. 43, 391-398.

Filgueira, R., Fernandez-Reiriz, M.J., Labarta, U. 2009. Clearance rate of the mussel *Mytilus galloprovincialis.* I. Response to extreme chlorophyll ranges. Ciencias Marinas. 35, 405-417.

Fitzpatrick, P.J., O´Halloran, J., Sheehan, D., Walsh, A.R., 1997. Assessment of a glutathione S-tranferase and related proteins in the gill and digestive gland of *Mytilus edulis* (L.), as potential organic pollution biomarkers. Biomarkers, 2, 51–6.

Fraga, F., 1981. Upwelling off the Galician coast, Northwest Spain. In: Richards FA, editor. Washington: Coastal Upwelling. American Geophysical Union, 176-182.

Fouillaron, P., Claquin, P., L'Helguen, S., Huonnic, P., Martin-Jézéquel, V., Masson, A., Longphuirt, S.N., Pondaven, P., Thouzeau, G., Leynaert, A., 2007. Response of a phytoplankton community to increased nutrient inputs: A mesocosm experiment in the Bay of Brest (France). J. Exp. Mar. Bio. Ecol. 351, 188-198. doi:10.1016/j.jembe.2007.06.009

Freites, L., Fernández-Reiriz, M.J., Labarta, U., 2003. Composición bioquímica y contenido energético del mejillón Mytilus galloprovincialis de origen submareal e intermareal: Influencia de las variables ambientales y de su origen. Ciencias Marinas. 29(4B), 603-619.

Frew, R.D., Hunter, K.A., Beyer, R., 1989. Cadmium in the dredge oyster, *Ostrea lutaria* -Dependence on age, body weight and distribution in internal organs. Mar. Poll. Bull. 20, 463-464.

Frouin, H., Pellerin, J., Fournier, M., Pelletier, E., Richard, P. 2007. Physiological effects of polycyclic aromatic hydrocarbons on soft-shell clam *Mya arenaria.* Aquatic toxicology, 82, 120-134

Funes, V., Alhama, J., Navas, J.I., López-Barea, J., Peinado, J., 2006. Ecotoxicological effects of metal pollution in two mollusc species from the Spanish South Atlantic litoral. Environ. Pollut. 139, 214–23.

Gabbott, P.A., 1975. Storage cycles in marine bivalve molluscs: an hypothesis concerning the relation between glycogen and gametogenesis. In: Barnes H, editor. Proceedings of the Ninth European Marine Biology Symposium. Aberdeen: Aberdeen University Press.

Gabbott, P.A., 1976. Energy metabolism. In: Marine mussels: their ecology and physiology. Bayne BL, editor. London: Cambridge University Press, 261-291.

Gabbott, P.A., 1983. Developmental and seasonal metabolic activities in marine molluscs. In: The Mollusca, volume 2: Environmental Biochemistry and Physiology. P.W. Hochachka (Ed.). Academic Press, pp 165-219.

Gabbott, P.A., Peek, K., 1991. Cellular biochemistry of the mantle tissue of the mussel *Mytilus edulis* L. Aquaculture. 94, 165-176.

Gabbott, P,A,, Whittle, M.A., 1986. Glycogen synthetase in the sea mussel *Mytilus edulis L*. II: Seasonal changes in glycogen content and glycogen synthetase activity in the mantle tissue. Comp. Biochem. Physiol. B. 83, 197-207.

Gabbott, P.A., Bayne, B.L., 1973. Biochemical effects of temperature and nutritive stress on *Mytilus edulis L*. J. Mar. Biol. Assoc. U.K. 53, 269–286.

Gagnaire, B., Thomas-Guyon, H., Renault, T., 2004. In vitro effects of cadmium and mercury on Pacific oyster, *Crassostrea gigas* (Thunberg), haemocytes. Fish Shellfish Immunol. 16, 501–12. doi:10.1016/j.fsi.2003.08.007

Gagnaire, B., Gay, M., Huvet, A., Daniel, J.Y., Saulnier, D., Renault, T. 2007. Combination of a pesticide exposure and a bacterial challenge: in vivo effects on immune response of Pacific oyster, *Crassostrea gigas* (Thunberg), Aquat. Toxicol. 84 (1), 92-102.

Gagné, F., Burgeot, T., Hellou, J., St-Jean, S., Farcy, E., Blaise, C., 2008. Spatial variations in biomarkers of *Mytilus edulis* mussels at four polluted regions spanning the Northern Hemisphere. Environ. Res. 107, 201–17. doi:10.1016/j.envres.2007.09.013

Gamliel, A., Afri, M., Frimer, A.A. 2008. Determining radical penetration of lipid bilayers with new lipophilic spin traps. Free Rad. Biol. Med. 44, 1394-1405. Doi:10.1111/j.freeradbiomed.2007.12.028

Geret, F., Burgeot, T., Haure, J., Gagnaire, B., Renault, T., Communal, P.Y., Samain, J.F. 2013. Effects of low-dose exposure to pesticide mixture on physiological responses of the Pacific oyster, *Crassostrea gigas*. Environ. Toxicol. 28 (12), 689-699, http://dx.doi.org/10.1002/tox.20764.

Giannapas, M., Karnis, L., Dailianis, S., 2012. Generation of free radicals in haemocytes of mussels after exposure to low molecular weight PAH components: immune activation, oxidative and genotoxic effects. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 155, 182–9. doi:10.1016/j.cbpc.2011.08.001

Gil, J., 2008. Macro and mesoscale physical patterns in the Bay of Biscay. J. Mar. Biol. Ass. UK. 88 (2), 217-225.

Goldberg, E.D. (1986) The mussel watch concept. Environ. Monitor. Assess. 7, 91-103.

Gomez-Gesteira, M., Beiras, R., Presa, P., Vilas, F., 2011. Coastal processes in northwestern Iberia, Spain. Continental. Shelf. Research. 31 (5), 367-375.

González-Quijano, A., Fumega, J., 1996. Determinación de congéneres individuales de bifenilos policlorados en organismos marinos. Inf. Téc. Insti. Esp. Oceanogr. 160, 1-27.

González-Fernández, C., Albentosa, M., Campillo, J.A., Viñas, L., Fumega, J., Franco, A., Besada, V., González-Quijano, A., Bellas, J., 2015a. Influence of mussel biological

variability on pollution biomarkers. Environ. Res. 137C, 14–31. doi:10.1016/j.envres.2014.11.015

González-Fernández, C., Albentosa, M., Campillo, J.A., Viñas, L., Romero, D., Franco, A., Bellas, J., 2015b. Effect of nutritive state on *Mytilus galloprovincialis* pollution biomarkers: Implications for large-scale monitoring programmes. Aquat. Toxicol. 167, 90–105. doi:10.1016/j.aquatox.2015.07.007

González-Fernández, C., Albentosa, M., Campillo, J. A, Viñas, D., Franco, A., Bellas, J., 2015c. Effect of mussel reproductive status on pollution biomarkers: Implications for large-scale monitoring programs. (Submitted)

Green, N.W., Schøyen, M., Øxnevad, S., Ruus, A., Høgåsen, T., Beylich, B., Håvardstun, J., Rogne, Å.K.G., Tveiten, L., 2012. Hazardous substances in fjords and coastal waters - 2011. Levels, trends and effects. Long-term monitoring of environmental quality in Norwegian coastal waters. NIVA. http://rapp.niva.no/symfoni/RappArkiv8.nsf/URL/451FAEE69D3C712AC1257B1F004D63EF/$FILE/6432-2012.pdf.

Gosling, E., 2003. Bivalve Molluscs. Biology, Ecology and Culture. Fishing News Books, Oxford, p. 443.

Griffiths, R.J., 1977. Reproductive cycles in littoral populations of *Choromytilus meridionalis* (Kr) and *Aulacomya ater* (Molina) with quantitative assessment of gamete production in the former. J. Exp. Mar. Biol. Ecol. 30, 53-71.

Griffin, J.J., Koide, M., Hodge, V., Goldberg, E.A., 1980. Estimating the ages of mussels by chemical and physical methods. In: Isotope Marine Chemistry, Goldberg, E.D., Horibe, Y. and Saruhashi, K., editors. , Rokakuho, Tokyo,193-209.

Griesbaum, K., Behr, A., Biedenkapp, D., Voges, H-W., Garbe, D., Paetz, C., Collin, G., Mayer, D. 2002. Hartmut Höke "Hydrocarbons" in Ullmann's. Encyclopedia of Industrial Chemistry. Wiley-VCH, Weinheim. doi:10.1002/14356007.a13_227

Grundy, M.M., Moore, M.N., Howell, S.M., Ratcliffe, N.A., 1996. Phagocytic reduction and effects on lysosomal membranes by polycyclic aromatic hydrocarbons, in haemocytes of *Mytilus edulis*. Aquat. Toxicol. 34, 273–290. doi:10.1016/0166-445X(95)00044-5

Gundersen, D.T., Miller, R., Mischler, A., Elpers, K., Mims, S.D., Millar, J.G., Blazer, V., 2000. Biomarker response and health of polychlorinated biphenyl- and chlordane-contaminated paddlefish from the Ohio river basin, U.S.A. Environ. Toxicol. Chem. 19 (9), 2275–2285.

Haberkorn, H., Lambert, C., Le Goïc, N., Quéré, C., Bruneau, A., Riso, R., Auffret, M., Soudant, P., 2014. Cellular and biochemical responses of the oyster *Crassostrea gigas*

to controlled exposures to metals and Alexandrium minutum. Aquat. Toxicol. 147, 158–67. doi:10.1016/j.aquatox.2013.12.012

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferase. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130-7139.

Hagger, J. A., Lowe, D., Dissanayake, A., Jones, M.B., Galloway, T.S., 2010. The influence of seasonality on biomarker responses in *Mytilus edulis*. Ecotoxicology 19, 953–62. doi:10.1007/s10646-010-0477-0

Halldórsson, H.P., Svavarsson, J., Granmo, A., 2005. The effect of pollution on scope for growth of the mussel (*Mytilus edulis* L.) in Iceland. Mar. Environ. Res.59, 47–64.

Hawkins AJS, Smith RFM, Tan SH, Yasin ZB. 1998. Suspension-feeding behaviour in tropical bivalve molluscs: *Perna viridis, Crassostrea belcheri, Crassostrea iradelei, Saccostrea cucculata* and *Pinctada margarifera*. Mar Ecol. Prog. Ser. 166, 173-185 .

Hawkins A.J.S., Fang, J.G., Pascoe, P.L., Zhang, J.H., Zhang, X.L., Zhu, M.Y., 2001. Modelling short-term responsive adjustments in particle clearance rate among bivalve suspension-feeders: separate unimodal effects of seston volume and composition in the scallop *Chlamys farreri* . J. Exp. Mar. Biol. Ecol. 262, 61-73.

Hawkins, A. J. S., Smith, R. F. M., Bayne, B.L., Héral, M. 1996. Novel observations underlying the fast growth of suspension–feeding shellfish in turbid environments: *Mytilus edulis.* Marine Ecology Progress Series, 131, 179–190.

Hawkins, A. J. S., Bayne, B. L., Bougrier, S., Héral, M., Iglesias, J.I.P., Navarro, E., Smith, R. F. M., Urrutia, M.B. 1998. Some general relationships in comparing the feeding physiology of suspension–feeding bivalve molluscs. J. Exp. Mar. Biol. Ecol. 219, 87-103.

Hégaret, H., Wikfors, G.H., Soudant, P., 2003. Flow cytometric analysis of haemocytes from eastern oysters, *Crassostrea virginica*, subjected to a sudden temperature elevation: II. Haemocyte functions: aggregation, viability, phagocytosis, and respiratory burst. J. Exp. Mar. Biol. Ecol. 293, 249-265. doi:10.1016/S0022-0981(03)00235-1

Hidalgo, M.C., Exposito, A., Palma, J.M. and Higuera, de la M. 2002. Oxidative stress generated by dietary Zn-deficiency: studies in rainbow trout (*Oncorhynchus mykiss*). Int. J. Biochem. Cell Biol. 34, 183–193.

Hildreth, D.I., Crisp, D.J., 1976. A corrected formula for calculation of filtration rate of bivalve molluscs in an experimental flowing system. J. Mar. Biol. Assoc. UK. 56, 111–120.

Hoferkamp, L., Hermanson, M.H., Muir, D.C.G. 2010. Current use pesticides in Arctic media; 2000-2007. Sci. Total Environ. 408, 2985–2994. doi:10.1016/j.scitotenv.2009.11.038

Honkoop, P.J.., Bayne, B.., Drent, J., 2003. Flexibility of size of gills and palps in the Sydney rock oyster *Saccostrea glomerata* (Gould, 1850) and the Pacific oyster *Crassostrea gigas* (Thunberg, 1793). J. Exp. Mar. Bio. Ecol. 282, 113–133. doi:10.1016/S0022-0981(02)00463-X

Howell, R., Grant, A.M., Maccoy, N.E.J., 1984. Effect of treatment with reserpine on the change in filtration rate of the *Mytilus edulis* subjected to dissolved copper. Mar, Pollut, Bull, 15, 436-439

Hulbert, A.J., 2007. Membrane fatty acids as pacemakers of animal metabolism. Lipids 42, 811–819.

Hummel, H., UitOudeGroeneveld, J.P., Nieuwenhuize, J., van Liere, J.M., Bogaards, R.H., DeWolf, L., 1989. Relationship between PCB concentrations and reproduction in mussels *Mytilus edulis*. Mar. Environ.l Res. 28, 489-493.

Hummel, H., Bogaards, R.H., Nieuwenhuize, J., De Wolf, L., Van Liere, J.M., 1990. Spatial and seasonal changes differences in the PCB content of the mussels *Mytilus edulis*. Sci. Total. Environ. 92, 155-163.

Ibarrola, I., Iglesias, J.I.P., Navarro, E., 1996. Differential absorption of biochemical components in the diet of the cockle *Cerastoderma edule*: enzymatic responses to variations in seston compositions. Can. J. Zool. 74, 1887–1897.

Ibarrola, I., Larretxea, X., Iglesias, J.I.P., Urrutia, M.B., Navarro, E., 1998a. Seasonal variation of digestive enzyme activities in the digestive gland and the crystalline style of the common cockle *Cerastoderma edule*. Comp. Biochem. Physiol. 121, 25–34.

Ibarrola, I., Navarro, E., Iglesias, J.I.P., 1998b. Short-term adaptation of digestive processes in the cockle *Cerastoderma edule* exposed to different food quantity and quality. J. Comp. Physiol. B 168, 32–40.

ICES 2006. Techniques in Marine Environmental Sciences. Biological effect of contaminants. Measurement of the scope for growth in blue mussels. Nº40. pp 34.

ICES 2007. Report of the ICES/OSPAR workshop on Integrated monitoring of contaminants and their Effect in Coastal and Open-sea Areas (WKIMON III). 16-18 January 2007.ICES Headquarters. ICES CM 2007/ACME:01.pp 209.

ICES 2008. Report of the Fourth ICES/OSPAR Workshop on Integrated Monitoring of Contaminants and their Effects in Coastal and Open Sea Areas (WKIMON IV), 5–7 February 2008. (ICES, Copenhagen, Denmark. ICES CM 2008/ACOM:49) pp. 71

ICES, 2013. Report of the ICES Working Group on Biological Effects of Contaminants (WGBEC). 10–15 March 2013, San Pedro del Pinatar, Spain. ICES CM 2013/SSGHIE:04. 1-37.

ICES, 2014. Report of the ICES Working Group on Biological Effects of Contaminants (WGBEC). 3-7 March 2014, ICES Headquarters, Copenhagen, Denmark.

Iglesias, J.I.P., Pérez-Camacho, A., Navarro, E., Labarta, U., Beiras, R., Hawkins, A.J.S., 1996. Microgeographic variability in feeding, absorption and condition of mussels (*Mytilus galloprovincialis* Lmk): a transplant experiment. J. Shellfish. Res.15, 673-680.

Irabien, M.J., Cearrreta, A., Leorri, E., Gómez, J., Viguri, J., 2008. A 130 year of pollution in the Suances estuary (southern Bay of Biscay): Implications for environmental management. Mar. Poll. Bull. 56, 1719-1727.

Irisarri, J., Fernández-Reiriz, M.-J., De Troch, M., Labarta, U., 2014. Fatty acids as tracers of trophic interactions between seston, mussels and biodeposits in a coastal embayment of mussel rafts in the proximity of fish cages. Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 172-173, 105–15. doi:10.1016/j.cbpb.2014.04.006

Itziou, A., Dimitriadis, V.K., 2013. Effect of starvation and hibernation on the values of five biomarkers of general and specific stress using the land snail *Eobania vermiculata.* Comp. Biochem. Physiol. Part B 165, 236–242. doi:10.1016/j.cbpb.2013.05.004

Jambeck, J.R., Geyer, R., Wilcox, C., Siegler, T.R., Perryman, M., Andrady, A., Narayan, R., Law, C.L. 2015. Plastic waste inputs from land into the ocean. Science Reports 347, 6223.

Jarque, S., Prats, E., Olivares, A., Casado, M., Ramón, M., Piña, B., 2014. Seasonal variations of gene expression biomarkers in *Mytilus galloprovincialis* cultured populations: Temperature, oxidative stress and reproductive cycle as major modulators. Sci. Total Environ. 499, 363–372. doi:10.1016/j.scitotenv.2014.08.064

Jing-Jing, M., Lu-Qing, P., Jing, L., Lin, Z. 2009. Effects of benzo [a] pyrene on DNA damage and histological alterations in gonad of scallop *Chlamys farreri.* Marine Environmental Research, 67: 47-52.

Jensen, J., 2012. Risk evaluation of five groups of persistent organic contaminants in sewage sludge. Environmental Project No. 1406. Danish Environmental Protection Agency

Jeong G and Cho S. 2007. Long-term effect of polycyclic aromatic hydrocarbon on physiological metabolisms of the Pacific oyster, *Crassostrea gigas*. Aquaculture 265 (2007) 343–350.

Johnson, P.T., 1980. Hemopoietic tissue, hemocytes, and hemolymph. In: Johnson, P.T. (Ed.), Histology of Blue Crab, Callinectes sapidus: A Model for the Decapod. Praeger, New York, pp. 267–307.

Jorgensen, E.H., Burkow, I.C., Foshaug, H., Killie, B., Ingebrigtsen, K., 1997. Influence of lipid status on tissue distribution of the persistent organic pollutant octachlorostyrene in Arctic charr (*Salvelinus alpinus*). Comp. Biochem. Physiol. 118C, 311–318.

Jørgensen, C. B. 1996. Bivalve filter feeding revisited. Mar. Ecol. Prog.Ser.142, 287-302.

Jørgensen, C.B., 1990. Bivalve Filter Feeding: Hydrodynamics, Bioenergetics, Physiology and Ecology. Olsen and Olsen, Fredensborg, Denmark. 144 pp.

Kaloyianni, M., Dailianis, S., Chrisikopoulou, E., Zannou, a., Koutsogiannaki, S., Alamdari, D.H., Koliakos, G., Dimitriadis, V.K., 2009. Oxidative effects of inorganic and organic contaminants on haemolymph of mussels. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 149, 631–639. doi:10.1016/j.cbpc.2009.01.006

Keith, L.H., W.A. Teillard. 1979. Priority pollutants I: a perspective view. Environ. Sci. Tech,13(4), 416-423.

Klionsky, D.J., Emr, S.D. 2000. Autophagy as a regulated pathway of cellular degradation. Science. 290 (5497), 1717-1721.

Kim, Y., Ashton-Alcox, K.A., Powell, E.N., 2006. Histological Techniques for Marine Bivalve Molluscs: Update. Silver Spring, MD. NOAA Technical Memorandum NOS NCCOS, 1-27.

Kimbrough, K.L., Johnson, W.E., Lauenstein, G.G., Christensen, J.D., Apeti, D.A., 2008. An assessment of two decades of contaminant monitoring in the nation´s coastal zone. In: Silver Spring, M.D.N.T.M., editot. NOOA, 1-105.

Klarić, S., Pavičič-Hamer, D., Lucu, C., 2004. Seasonal variations of arsenic in mussels *Mytilus galloprovincialis*. Helgolland. Mar. Res. 58(3), 216-220.

Kluytmans, J. H., Brands, F., Zandee, D. I. 1988. Interactions of cadmium with the reproductive cycle of *Mytilus edulis L.* Mar. Environ. Res. 24: 189–192

Knauer, J., Southgate, P. C. 1999. A review of the nutritional requirements of bivalves and the development of alternative and artificial diets for bivalve aquaculture. Rev. Fish. Sci. 7(3-4), 241-280.

Kraak, M. H. S., Stuijfzand, S. C., Admiraal, W. 1999. Short-term ecotoxicity of a mixture of five metals to the zebra mussel *Dreissena polymorpha*. B. Environ. Contam. Tox. 63(6), 805-812.

Kumar, A., Malik, A.K., Tewary, D.K., Singh, B., 2008. Gradient HPLC of antibiotics in urine, ground water, chicken muscle, hospital wastewater, and pharmaceutical samples using C-18 and RP-amide columns. J. Sep. Sci. 31, 294–300. doi:10.1002/jssc.200700373

Labarta, U., Fernández-Reiriz, M.J., Babarro, J.M.F., 1997. Differences in physiological energetic between intertidal and raft cultivated mussels *Mytilus galloprovincialis*. Mar. Ecol. Prog. Ser. 152, 167–73.

Lacroix, C., Le Cuff, N., Receveur, J., Moraga, D., Auffret, M., Guyomarch, J., 2014. Development of an innovative and "green" stir bar sorptive extraction thermal desorption gas chromatography tandem mass spectrometry method for quantification of polycyclic aromatic hydrocarbons in marine biota. J. Chromatogr. A 1349, 1–10. doi:10.1016/j.chroma.2014.04.094

Lam, P.K.S., Gray, J.S., 2003.The use of biomarkers in environmental monitoring programmes. Mar. Pollut. Bull. 46, 182-186.

Lam, P.K.S., 2009. Use of biomarkers in environmental monitoring. Ocean and coastal management. 52, 348–354.

Lambert, C., Soudant, P., Choquet, G., Paillard, C., 2003. Measurement of *Crassostrea gigas* hemocyte oxidative metabolism by flow cytometry and the inhibiting capacity of pathogenic vibrios. Fish. Shellfish. Immunol. 15, 225-240.

Lavín, A., Valdés, L., Gil, J., Moral, M., 1998. Seasonal and interannual variability in properties of surface water off Santander (Bay of Biscay) (1991-1995). Oceanologica. 21 (2), 179-190.

Law, R.J., Kelly, C., 2004. The impact of the "Sea Empress" oil spill. Aquatic. Living. Resour. 17, 389-394.

Law, K. L., Thompson, R. C. 2014 Microplastics in the seas. Science 345, 144-145.

Lee, R.F., Keeran, W.S., Pickwell, G.V., 1988. Marine invertebrate glutathione S-transferase: purification, characterisation and induction. Mar, Environ, Res, 24, 97-100.

Lehane, C., Davenport, J. 2006. A 15 month study of zooplankton ingestion by farmed mussels (*Mytilus edulis*) in Bantry Bay, Southwest Ireland. Estuar. Coast. Shelf Sci. 67, 645–652.

Leiniö, S., Lehtonen, K.K., 2005. Seasonal variability in biomarkers in the bivalves Mytilus edulis and Macoma balthica from the northern Baltic Sea. Comp. Biochem. Physiol. C. 140, 408-421.

Levine, B., 2005. Eating oneself and uninvited guests: Autophagy-related pathways in cellular defense. Cell. 120, 159-162.

Lionetto, M.G., Caricato, R., Giordano, M.E., Erroi, E., Schettino, T., 2012. Carbonic anhydrase as pollution biomarker: An ancient enzyme with a new use. Int. J. Environ. Res. Public. Health. 9, 3965-3977.

Liu, D., Pan, L., Li, Z., Cai, Y., Miao, J., 2014. Metabolites analysis, metabolic enzyme activities and bioaccumulation in the clam *Ruditapes philippinarum* exposed to benzo[a]pyrene. Ecotoxicol. Environ. Saf. 107, 251–259. doi:10.1016/j.ecoenv.2014.06.024

Livingstone, D. R. 1985. Responses of the detoxication/toxication enzyme systems of mollusc to organic pollutants and xenobiotics. Mar. Pollut. Bull. 16, 158.

Livingstone, D.R., Pipe R.K. 1992. Mussels and environmental contaminants: molecular and cellular aspects. In: E. Gossling (Eds). Development in aquaculture and fishery science. Elsevier Pub. Co., Amsterdam. 425-456.

Livingstone, D.R., García Martínez, P., Michel, X., Narbonne, J.F., ÓHara, S., Ribera, D., Winston, G.W., 1990. Oxyradical production as a pollutant-mediated mechanism of toxicity in the common mussel, *Mytilus edulis* L., and other molluscs. Funct. Ecol., 415-424.

Livingstone, D.R., 1991. Organic xenobiotic metabolism in marine invertebrates. Adv. Comp. Environ. Physiol. 7, 45–185.

Livingstone, D.R., Donkin, P., Walker, C.H., 1992. Pollutants in marine ecosystems: an overview. In: Walker, C.H., Livingstone, D.R. (Eds.), Persistent Pollutants in Marine Ecosystems, SERAC Special Publication Series. Pergamon Press, pp. 235–263

Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar. Pollut. Bull. 42, 656–66.

Llope, M., Anadón, R., Álvarez-Ossorio, M., Valdés, L., Varela, M., 2003. Zooplankton biomass timing with temperature in South Bay of Biscay. 3rd International Zooplankton Production Symposium, Gijón.

Lotufo, G.R. 1998. Bioaccumulation of sediment-associated fluoranthene in benthic copepods: uptake, elimination and biotransformation. Aquat. toxicol. 44, 1-15.

Lowe, D.M., Moore, M.N., Clark, K.R., 1981. Effects of oil on digestive cells in mussels: quantitative alterations in cellular and lysosomal structure. Aquat. Toxicol. 12, 213–226.

Lowe, D.M., Pipe, R.K., 1986a. Hydrocarbon exposure in mussels: a quantitative study of the responses in the reproductive and nutrient storage cell systems. Aquatic Toxicology 8, 265–272.

Lowe, D.M., Pipe, R.K., 1986b. Cellular reponses in the mussel *Mytilus edulis* following exposure to diesel oil emulsion: Reproductive and nutrient storage cells. Mar. Environ. Res. 17, 234-237.

Lowe, D.M., Pipe, R.K., 1987. Mortality and quantitative aspects of storage cell utilization in mussels, *Mytilus edulis*, following exposure to diesel oil hydrocarbons. Mar. Environ. Res. 22, 243–251.

Lowe, D.M., Pipe, R.K., 1994. Contaminant induced lysosomal membrane damage in marine mussel digestive cells: an in vitro study. Aquat. Toxicol. 30, 357–365. doi:10.1016/0166-445X(94)00045-X

Lowe, D.M., Soverchia, C., Moore, M.N., 1995. Lysosomal membrane responses in the blood and digestive cells of mussels experimentally exposed to fluoranthene. Aquat.Toxicol. 33, 105–112.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 266-275.

Lushchak, V., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat, Toxicol, 101, 13-30.

Lubet, P., 1959. Recherches sur le cycle sexuel et l´émission des gametes chez les mytilides et les pectinides (Mollusques bivalves). Rev, Trav, Inst, Pêches, Marit, 23(4), 386-548.

Lavín, A., Valdés, L., Sánchez, F., Abaunza, P., Punzón, A., Bellas, J., et al. 2012. Demarcación marina Noratlántica. Parte I. Marco General. In: Ministerio de Agricultura, Alimentación y Medio Ambiente, editor. Estrategias Marinas: Evaluación inicial, Buen Estado Ambiental y Objetivos Ambientales. NIPO: 280-12-175-8; Madrid; 2012. http://www.magrama.gob.es/es/costas/temas/estrategias-marinas.

Leon, V.M., Gómez-Parra, A., González-Mazo, E., 2004. Biodegradation of linear alkylbenzene sulfonates and their degradation intermediates in seawater. Environ. Sci. Technol. 38, 2359–67. doi:10.1021/es034813

MacDonald, B.A., Ward, J. E. 1994. Variation in food quality and particle selectivity in the sea scallop *Placopecten magellanicus* (Mollusca: Bivalvia), Mar. Ecol. Prog.Ser. 108, 251-264.

Mallet, A.L., Carver C.E.A., Coffen, S.S., Freeman, K.R., 1987. Winter growth of the blue mussel Mytikus edulis L.: importance of stock and site. J. Exp. Mar. Biol. Ecol. 108, 217-228.

Manduzio, H., Monsinjon, T., Galap, C., Leboulenger, F., Rocher, B., 2004. Seasonal variations in antioxidant defences in blue mussels *Mytilus edulis* collected from a

polluted area: Major contributions in gills of an inducible isoform of Cu/Zn-superoxide dismutase and of glutathione S-transferase. Aquat. Toxicol. 70, 83–93. doi:10.1016/j.aquatox.2004.07.003

Manduzio, H., Rocher, B., Durand, F., Galap, C., Leboulenger, F., 2005. The point about oxidative stress in molluscs 91–104.

Mantel, N., Valand, R.S., 1970. A technique of nonparametric multivariate analysis. Biometrics. 26, 547-558.

Mao, J. S., Cao, J., & Graedel, T. E. 2009. Losses to the environment from the multilevel cycle of anthropogenic lead. Environ. Poll. *157*(10), 2670-2677.

Maria VL & Bebianno MJ. 2011. Antioxidant and lipid peroxidation responses in *Mytilus galloprovincialis* exposed to mixtures of benzo(a)pyrene and copper. Comp. Bio. Physiol. Part C:Toxicol. Pharmacol.154, 56-63.

Marsh, J.B., Weinstein, D.B., 1966. Simple charring method for determination of lipids. J. Lipid. Res. 7(4), 574-6.

Marie, D., Partensky, F., Vaulot, D., Brussaard, C.P.D., 1999. Enumeration of phytoplankton, bacteria, and viruses in marine samples, in: Robinson, J.P., Darzynkiewicz, Z., Dean, P.N., Orfao, A., Rabinovitch, P., Stewart, C., Tanke, H.J., Wheeless, L. (Eds.), Current Protocols in Cytometry. John Wiley & Sons, Inc., New York. N.Y., pp. 11.11.11–11.11.15.

Martínez-Álvarez, R., Morales, A.E., Sanz, A., 2005. Antioxidant defenses in fish: Biotic and abiotic factors. Reviews in Fish Biology and Fisheries. 15, 75-88.

Marsh, J.B., Weinstein, D.B., 1966. Simple charring method for determination of lipids. J. Lipid Res. 7, 574–576.

Marty, Y., Delaunay, F., Moal, J., Samain, J.F., 1992. Changes in the fatty acid composition of *Pecten maximus (L.)* during larval development. J. Exp. Mar. Biol. Ecol. 163, 221–234.

Mazière, C., Conte, M.A., Degonville, J., Ali, D., Mazière, J.C., 1999. Cellular enrichment with polyunsaturated fatty acids induces an oxidative stress and activates the transcription factors AP1 and NFkB. Biochem. Biophys. Res. Commun. 265, 116–122.

McCormick-Ray, M.G., 1987. Haemocytes of *Mytilus edulis* affected by Prudhoe Bay crude oil emulsion. Mar. Environ. Res. 17, 107–122.

McDowell, J.E., Lancaster, B. a., Leavitt, D.F., Rantamaki, P., Ripley, B., 1999. The effects of lipophilic organic contaminants on reproductive physiology and disease processes in marine bivalve molluscs. Limnol. Oceanogr. 44, 903–909. doi:10.4319/lo.1999.44.3_part_2.0903

McVeigh, A., Moore, M., Allen, J.I., Dyke, P., 2006. Lysosomal responses to nutritional and contaminant stress in mussel hepatopancreatic digestive cells: a modelling study. Mar. Environ. Res. 62 Suppl, S433–8. doi:10.1016/j.marenvres.2006.04.021

Matozzo, V., Ballarin, L., Pampanin, D. M., & Marin, M. G. 2001. Effects of copper and cadmium exposure on functional responses of hemocytes in the clam, *Tapes philippinarum*. Arch. Environ. Contam. Toxicol. 41(2), 163-170.

Matozzo, V., Gallo, C., Marin, M.G., 2011. Can starvation influence cellular and biochemical parameters in the crab *Carcinus aestuarii*? Mar. Environ. Res. 71, 207–12. doi:10.1016/j.marenvres.2011.01.004

Menezes S. Soares A. Guilhermino L. Peck M. 2006. Biomarker responses of the estuarine brown shrimp *Crangon crangon L.* to non-toxic stressors: Temperature, salinity and handling stress effects. J. of Exp. Mar. Biol.Ecol. 335, 114–122

Menone, M.L., Aizpún de Moreno, J.E., Moreno, V.J., Lanfranchi, A.L., Metcalfe, T.L., Metcalfe, C.D., 2000. PCBs and organochlorines in tisúes of silverside (*Odontesthes bonariensis*) from coastal lagoon in Argentina. Arch. Environ. Contam. Toxicol. 38 (2), 202–208

Molina, R., 1972. Contribución al estudio del *upwelling* frente a la costa noroccidental de la Península Ibérica. Bol. Inst. Esp. Oceanogr. 152, 0-39.

Moller, M., Botti, H., Batthyany, C., Rubbo, H., Radi, R., Dnicola, A. 2005. Direct measurement of nitric oxide and oxigen partitioning into liposomes and low density lipoprotein. J. Biol. Chem. 280, 8850-8854.

Morales, A., Pérez-Jiménez, A., Hidalgo, M.C., Abellán, E., Cardenete, G., 2004. Oxidative stress and antioxidant defenses after prolonged starvation in *Dentex dentex* liver. Comp. Bio. Physiol. C. 139, 153-161.

Moore, M.N., 1985. Cellular responses to pollutants. Mar. Pollut. Bull. 16, 134–139.

Moore, M.N., Lowe, D.M., Moore, S.L., 1979. Induction of lysosomal desta- bilisation in marine bivalve molluscs exposed to air. Mar. Biol. Lett. 1, 47–57.

Moore, M.N., Halton,D.W., 1973. Histochemical changes in the digestive gland of *Lymnaea truncatula* infected with *Fasciola hepatica*. Z. Parasitkde 43, 1–16.

Moore, M.N., 2004. Diet restriction induced autophagy: A lysosomal protective system against oxidative- and pollutant-stress and cell injury. Mar. Environ. Res. 58(2–5), 603-607.

Moore, M.N., Allen, J.I., Mcveigh, A., Shaw, J., 2006a. Lysosomal and Autophagic Reactions as Predictive Indicators Addenda ES SC ACKNOWLEDGEMENTS 217–220.

Moore, M.N., Allen, J.I., Somerfield, P.J., 2006b. Autophagy: role in surviving environmental stress. Mar. Environ. Res. 62 Suppl, S420–5. doi:10.1016/j.marenvres.2006.04.055

Moore, M.N., Kohler, A., Lowe, D., Viarengo, A., 2008. Lysosomes and autophagy in aquatic animals., 1st ed, Methods in enzymology. Elsevier Inc. doi:10.1016/S0076-6879(08)03233-3

Moore, M.N., Viarengo, A., Donkin, P., Hawkins, A.J.S., 2007. Autophagic and lysosomal reactions to stress in the hepatopancreas of blue mussels. Aquatic. Toxicol. 84 (1), 80-91.

Morales, A.E., Pérez-Jiménez, A., Hidalgo, M.C., Abellán, E., Cardenete, G., 2004. Oxidative stress and antioxidant defenses after prolonged starvation in *Dentex dentex* liver. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 139, 153–61. doi:10.1016/j.cca.2004.10.008

Mubiana, V.K., Vercauteren, K., Blust, R., 2006. The influence of body size, condition index and tidal exposure on the variability in metal bioaccumulation in *Mytilus edulis*. Environ. Pollut. 144, 272–9. doi:10.1016/j.envpol.2005.12.017

Muñoz, P., Meseguer, J., Esteban, M.A., 2006. Phenoloxidase activity in three commercial bivalve species. Changes due to natural infestation with *Perkinsus atlanticus*. Fish Shellfish Immunol. 20, 12–9. doi:10.1016/j.fsi.2005.02.002

Nakata, H., Shinohara, R., Nakazawa, Y., Isobe, T., Sudaryanto, A., Subramanian, A., Tanabe, S., Zakaria, M.P., Zheng, Paul, G.J., Lam, K.S., Kim, E.Y., Min, B., We, S., Viet, P.H., Tana, T.S., Prudente, M., Frank, D., Lauenstein, G., Kanna, K., 2012. Asia–Pacific mussel watch for emerging pollutants: Distribution of synthetic musks and benzotriazole UV stabilizers in Asian and US coastal waters. Mar. Poll. Bull. 64 (10), 2211-2218.

Nahrgang, J., Camus, L., Broms, F., Christiansen, J.S., Hop, H., 2010. Seasonal baseline levels of physiological and biochemical parameters in polar cod (*Boreogadus saida*): Implications for environmental monitoring. Mar. Poll. Bull. 60, 1336-1345.

Nahrgang, J., Brooks, S.F., Evenset, A., Camus, L., Jonsson, M., Smith, T.J., Lukina, J., Frantzen, M., Giarratano, E., Renaud, P.E., 2013. Seasonal variation in biomarkers in blue mussel (*Mytilus edulis*), Iceland scallop (*Chlamys islandica*) and Atlantic cod

(*Gadus morhua*)- Implications for environmental monitoring in the Barents Sea. Aquat. Toxicol. 127, 21-35.

Naudi, A., Jové, M., Ayala, V., Portero-Otín, M., Barja G., Pamplona, R., 2013. Membrane lipid unsaturation as physiological adaptation to animal longetivy. Front. Physiol. 4, 1-13. Doi:10.3389/fphys.2013.00372

Navarro, E., 1996. The effect of diets of phytoplankton and suspended bottom material on feeding and absorption of raft mussels (*Mytilus galloprovincialis Lmk*) 198, 175–189.

Navarro, E., Iglesias, J.I.P., Larrañaga, A., 1989. Interannual variation in the reproductive cycle and biochemical composition of the cockle *Cerastoderma edule* from Mundaca Estuary (Biscay, North Spain). Mar. Biol. 101, 503-511.

Navarro, E., Iglesias, J.I.P., 1993. Infaunal filter-feeder bivalves and the physiological response to short-term fluctuations in food availability and composition, In: Dame, R.F. (Eds.), Bivalve Filter Feeders in Estuarine and Coastal Ecosystem Processes. Springer, Berlin/Heidelberg, pp. 25-56.

Navarro, J.M., Labarta, U., Fernández-Reiriz, M.J., Velasco, A., 2003. Feeding behavior and differential absorption of biochemical components by the infaunal bivalve *Mulinia edulis* and the epibenthic *Mytilus chilensis* in response to changes in food regimes. J. Exp. Mar. Biol. Ecol. 287, 13-35.

Newell, R.I.E., Hilbish, T.J., Koehn, R.K., Newell, C.J., 1982. Temporal variation in the reproductive cycle of *Mytilus edulis* L (Bivalvia, Mytilidae) from localities on the east coast of the United States. Biol. Bull. 162, 299-310.

Newell, R.E., 1989. Species profiles: life histories and environmental requirements of coastal fishes and invertebrates (North and Mid Atlantic) – blue mussel. US Army Corps of Engineers, Report TR EL-82-4.

Nicholson, S. 2002. Ecophysiological aspects of cardiac activity in the subtropical mussel *Perna viridis (L)* (Bivalvia: Mytilidae). J.Exp. Mar. Biol. Ecol. 267, 207-222.

Nordberg, J., Arnér, S.J., 2001. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. Free Radic. Biol. Med. 31, 1287-1312.

Oliva, M., Vicente, J.J., Gravato, C., Guilhermino, L., Galindo-Riaño, M.D., 2012. Oxidative stress biomarkers in Senegal sole, *Solea senegalensis*, to assess the impact of heavy metals pollution in a Huelva estuary (SW Spain): Seasonal and spatial variation. Ecotoxicol. Environ. Saf. 75, 151-162.

Oliveira, P.B., Moita, T., Silva, A., Monteiro, I.T., Palma, A.S. 2009. Summer diatom and dinoflagellate blooms in Lisbon Bay from 2002 to 2005: Pre-conditions inferred from wind and satellite data. Prog. Oceanogr. 83, 270-277.

Olsen, R.E., Henderson, R.J. 1997. Muscle fatty acid composition and oxidative stress indices of Arctic charr, *Salvelinus alpinus* (L.), in relation to dietary polyunsaturated fatty acid levels and temperature. Aquacult. Nutr. 3, 227– 238.

Orban, E., Di Lena, G., Navigato, T., Casini, I., Marzetti, A., Caproni, R., 2010. Seasonal changes in meat content, condition index and chemical cmposition of mussels (*Mytilus galloprovincialis*) cultured in two different Italian sites. Food Chemistry, 77(1), 57-65.

Ortiz-Zarragoitia M & Cajaraville MP. 2006. Biomarkers of exposure and reproduction-related effects in mussels exposed to endocrine disruptors. Arch. Environ. Contam. Toxicol. 50, 361-369

Ortiz-Zarragoitia, M., Garmendia, L., Barbero, M.C., Serrano, T., Marigómez, I., Cajaraville, M.P., 2011. Effects of the fuel oil spilled by the Prestige tanker on reproduction parameters of wild mussel populations. J. Environ. Monit. 13, 84–94. doi:10.1039/c0em00102c

OSPAR Commission. 2010a. JAMP guidelines for monitoring contaminants in biota. Update 2010 (Agreement 1999–02). OSPAR Comission, London.

OSPAR Commission. 2010b. Quality Status Report 2010. OSPAR Commission, London.

OSPAR Commission. 2012a. Cemp 2011 Assessment Report. Monitoring Assessment Series. OSPAR Commission, London.

OSPAR Commission. 2012b. Environmental Assessment Criteria (EACs) for hazardous substances in the marine environment: OSPAR contribution to addressing the challenge of assessing chemical quality across all marine waters. OSPAR Commission, London.

Othman, H.B., Leboulanger, C., Le Floc'h, E, Mabrouk, H.H., Hlaili, A.S. 2012. Toxicantity of benz(a)anthracene and fluoranthene to marine phytoplankton in culture: Does cell size really matter?. J. Hazard. Mater. 243, 204-211.

Ospina-Alvarez, N., Caetano, M., Vale, C., Santos-Echeandía, J., Prego, R., 2014. Prevalence of tide-induced transport over other metal sources in a geologically enriched températe estuarine zone (NW Iberian Peninsula). J. Geochem. Exp. 140, 46-55.

Palais, F., Dedourge-Geffard, O., Beaudon, a, Pain-Devin, S., Trapp, J., Geffard, O., Noury, P., Gourlay-Francé, C., Uher, E., Mouneyrac, C., Biagianti-Risbourg, S., Geffard, a, 2012. One-year monitoring of core biomarker and digestive enzyme responses in transplanted zebra mussels (Dreissena polymorpha). Ecotoxicology 21, 888–905. doi:10.1007/s10646-012-0851-1

Pamplona, R., Costantini, D., 2011. Molecular and structural antioxidant defenses against oxidative stress in animals. Am. J. Physiol. Regul. Integr. Comp. Physiol. 301, R843–63. doi:10.1152/ajpregu.00034.2011

Pan, L., Ren, J., Liu, J., 2005. Effects of benzo(k)fluoranthene exposure on the biomarkers of scallop *Chlamys farreri*. Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 141, 248–256. doi:10.1016/j.cca.2005.07.005

Parrish, C., Abrajano, T., Budge, S., Helleur, R., Hudson, E., Pulchan, K.E.A. 2000. Lipid and Phenolic biomarkers in marine ecosystems: Analyses and applications. Springer. Berlin.

Parrish, C.C., Thompson, R.J., Deibel, D., 2005. Lipid classes and fatty acids in plankton and settling matter during the spring bloom in a cold ocean coastal environment. Mar. Ecol. Prog. Ser. 286, 57-68.

Pascual, P., Pedrajas, J.R., Toribio, F., López-Barea, J., Peinado, J., 2003. Effect of food deprivation on oxidative stress biomarkers in fish (*Sparus aurata*). Chem-Biol. Int. 145, 191-199.

Pérez-Camacho, A., Labarta, U., Beiras, R., 1995. Growth of mussels (Mytilus edulis galloprovincialis) on cultivation rafts: influence of seed source, cultivation site and phytoplankton availability. Aquaculture 138, 349-362.

Pérez Camacho, A., Labarta, U., Navarro, E., 2000. Energy balance of mussels *Mytilus galloprovincialis:* the effect of length and age. Mar. Ecol., Prog. Ser. 199, 149–158.

Perpétua, M., Scarlet, J., Pálmar, H., Granmo, Å., 2015. Scope for growth and condition index in the clam *Meretrix meretix (L .)* as biomarkers of pollution in Espírito Santo Estuary , Mozambique. Reg. Stud. Mar. Sci. 1, 63–71. doi:10.1016/j.rsma.2015.03.002

Peterson, R.A., 2000. A meta-analysis of variance accounted for and factor loadings in exploratory factor analysis. Marketing Lett. 11 (3), 261-275.

Peterson, C.H., Beal, B.F., 1989. Bivalve growth and higher order interactions: Importance of density, site, and time. Ecology 70(5), 1390-1404.

Peteiro, L.G., Labarta, U., Fernández-Reiriz, M.J., Álvarez-Salgado, X.A., Filgueira, R., Piedracoba, S., 2011. Influence of intermittent-upwelling on *Mytilus galloprovincialis* settlement patterns in the Ría de Ares-Betanzos. Mar. Ecol. Prog. Ser. 443, 111-127.

Pichaud, N., Pellerin, J., Fournier, M., Gauthier-Clerc, S., Rioux, P., Pelletier, É. 2008. Oxidative stress and immunologic responses following a dietary exposure to PAHs in *Mya arenaria*. Chem. Central J. 2(1), 23.

Pieters, H., Kluytmans, J.H.,Zandee, D.I., Cadée, C.C. 1980. Tissue composition and reproduction of *Mytilus edulis* in relation to food availability. Neth. J. Sea. Res. 14, 349-361.

Pipe R.K. 1987. Mortality and quantitative aspects of storage cell utilization in mussels, *Mytilus edulis*, following exposure to diesel oil hydrocarbons. Mar. Environ. Res. 22: 243–251.

Pipe, R.K. 1990. Hydrolytic enzymes associated with the granular haemocytes of the marine mussel *Mytilus edulis*. Cell. Tissue. Res. 261, 261-268.

Pipe, R.K., Porte, C., Livingstone, D.R. 1993. Atioxidant enzymes associated with the blood cells and haemolymph of the mussels Mytilus edulis. Fish. Shellfish. Immun. 3, 221-233.

Pipe, R.K., Coles, J.A. 1995. Environmental contaminations influencing immune function in marine bivalve molluscs. Fish .Shellfish. Immunol. 5, 581–595.

Pipe, R., Coles, J., Thomas, M., Fossato, V., Pulsford, A., 1995. Evidence for environmentally derived immunomodulation in mussels from the Venice Lagoon. Aquat. Toxicol. 32, 59–73. doi:10.1016/0166-445X(94)00076-

Porte, C., Sole, M., Albaiges, J., Livingstone, D.R., 1991. Responses of mixed-function oxygenase and antioxidase enzyme system of *Mytilus sp*. to organic pollution. Comp. Biochem. Physiol. C 100, 183– 186.

Porte, C., Biosca, X., Pastor, D., Solé, M., Albaigés, J., 2000. The aegean Sea oil spill. Temporal study of the hydrocarbons accumulation in bivalves. Environ Scir Technol 34, 5067-5075.

Porte C, Biosca X, Pastor D, Solé M, Albaigés J (2001a). The integrated use of chemical analysis, cytochrome P450 ahd stress proteins in mussels to assess pollution along the Galician coast (NW Spain). Environ Pollut 112: 261-268

Pörtner, H.O., 2002. Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. Comp. Bio. Physiol. Part. A. 132, 739-761.

Power, A., Sheehan, D., 1996. Seasonal variation in the antioxidant defence systems of gill and digestive gland of the blue mussel, Mytilus edulis. Comp. Biochem. Physiol. C. 114, 99-103.

Prevodnik, A., Lilja, K., Bollner, T., 2007. Benzo[a]pyrene up-regulates the expression of the proliferating cell nuclear antigen (PCNA) and multixenobiotic resistance

polyglycoprotein (P-gp) in Baltic Sea blue mussels (*Mytilus edulis* L.). Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 145, 265–74. doi:10.1016/j.cbpc.2006.12.014

Prins, T. C., Small, A. C., Pouwer, A. J. 1991. Selective ingestion of phytoplankton by the bivalves *Mytilus edulis L.* and *Cerastoderma edule (L.).* Hydrobiological Bulletin, 25, (1), 93-100.

Prosser CL. Physiological Adaptation. Washington, DC: American Physiological Society, 1958:158.

Ramos-Martínez, J.I., Rodrigez-Bartolomé, T., Vázquez-Pernas, R., 1983. Purification and properties of glutathion reductase from hepatopáncreas of *Mytilus edulis L*. Comp. Biochem. Physiol. 75B, 689-692.

Regoli, F., 1998. Trace metals and antioxidant enzymes in gills and digestive gland of the Mediterranean mussel *Mytilus galloprovincialis*. Arch. Environ. Contam. Toxicol. 34, 48-63.

Regoli, F., Principato, G., 1995. Glutathione, glutathione-dependent and antioxidant enzymes in mussel, *Mytilus galloprovincialis*, exposed to metals under field and laboratory conditions: implications for the use of biochemical biomarkers. Aquat. Toxicol. 64, 31-143.

Regoli, F., Giuliani, M.E., 2014. Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. Mar. Environ. Res. 93, 106-117.

Ren, X., Pan, L., Wang, L., 2014. Toxic effects upon exposure to benzo[a]pyrene in juvenile white shrimp *Litopenaeus vannamei*. Environ. Toxicol. Pharmacol. 39, 194-207. doi:10.1016/j.etap.2014.08.006

Renault, T., 2015. Immunotoxicological effects of environmental contaminants on marine bivalves. Fish Shellfish Immunol. 46, 88–93. doi:10.1016/j.fsi.2015.04.011

Richir, J., Gobert, S., 2014. The effect of size, weight, body compartment, sex and reproductive status on the bioaccumulation of 19 trace elements in rope-grown *Mytilus galloprovincialis*. Ecol. Indic. 36, 33-47.

Riisgard, H.U., 2001. On measurement of filtration rates in bivalves—the stony road to reliable data: review and interpretation. Mar. Ecol., Prog. Ser. 211, 275–291

Riisgård, H.U., Pleissner, D., Larsen, P.S., Lundgreen, K. 2013. Growth of mussels *Mytilus edulis* at algal (*Rhodomonas salina*) concentrations below and above saturation level for reduced filtration rate. Mar Biol Res. 9, 1005-1017

Roberts, D.M., Aaron, C.K. 2007. Management of acute organophosphorus pesticide poisoning. BMJ. 334(7594), 629-634.

Rodhouse, P.G., Roden, C.M., Burnell, G.M., Hensey, M.P., McMahon, T., Ottway, B., Ryan, T.H., 1984. Food resource, gametogenesis and growth of *Mytilus edulis* on the shore and in suspended culture: Wary Harbour, Ireland. J. Mar. Biol. Ass. UK. 64,513-530.

Rodrigues, a P., Lehtonen, K.K., Guilhermino, L., Guimarães, L., 2013. Exposure of *Carcinus maenas* to waterborne fluoranthene: accumulation and multibiomarker responses. Sci. Total Environ. 443, 454–63. doi:10.1016/j.scitotenv.2012.10.077

Rodríguez de la Rúa, A., Arellano, J. M., González de Canales, M. L., Blasco, J., Sarasquete, C. 2005. Acumulación de cobre y alteraciones histopatológicas en el ostión *Crassostrea angulata*. Ciencias Marinas, 31(3), 455-466.

Roose P. 2010. Volatile organic compounds and related microcontaminants. Lambrecht Academic Publishing AG & CO. KG, Saarbrücken, Germany ISBN 978-3-8383-2310, 226 pp.

Rouillon, G., Navarro, E., 2003. Differential utilization of species of phytoplankton by the mussel Mytilus edulis. Acta Oecologica 24, S299-S305. doi:10.1016/S1146-609X(03)00029-8

Rueda-Jasso, R., Conceicao, L.E.C., Dias, J., De Coen, W., Gomes, E., Rees, J.F., Soares, F., Dinis, M.T., Sorgeloos, P. 2004. Effect of dietary non-protein energy levels on condition and oxidative status of Senegalese sole (*Solea senegalensis)* juveniles. Aquaculture 231, 417–433.

Rueda, J., Smaal, A., 2004. Variation of the physiological energetic of the bivalve *Spisula subtruncata* (da Costa, 1778) within an annual cycle. J. Exp. Mar. Biol. Ecol. 301, 141–157.

Sami, S., Faisal, M., Huggett, R.J., 1992. Effects of laboratory exposure to sediments contaminated with polycyclic aro- matic hydrocarbons on the hemocytes of the American oyster *Crassostrea virginica* . Mar. Environ. Res. 35, 131-135.

Schmidt, W., Power, E., Quinn, B., 2013. Seasonal variations of biomarker responses in the marine blue mussel (*Mytilus* spp.). Mar. Poll. Bull. 74, 50-55.

Seed, R., 1976. Ecology. In: Bayne BL, editor. Marine Mussels: their ecology and physiology. London: Cambridge University Press, 13–66.

Segovia-Zavala, J.A., Delgadillo-Hinojosa, F., Vidal-Talamantes, R., Munoz-Barbosa, A., Gutierrez-Galindo, E.A., 2003. *Mytilus californianus* transplanted as upwelling bioindicators to two areas off Baja California, Mexico. Ciencias Marinas. 29, 665-675.

Sericano, J.L., Wade, T.L., Sweet, S.T., Ramirez, J., Lauenstein, G.G., 2014. Temporal trends and spatial distribution of DDT in bivalves from the coastal marine environments of the continental United States, 1986–2009. Mar. Poll. Bull. 81, 303-316.

Sheehan, D., Meade, G., Foley, V.M., Dowd, C.A., 2001. Structure, function and evolution of glutathione transferase for classification of non-mammalian members of an ancient superfamily. Biochem. J. 360, 1-16.

Sheehan, D., Power, A., 1999. Effects of seasonality on xenobiotic and antioxidant defence mechanism of bivalve molluscs. Comp. Biochem. Physiol. 9, 123-193.

Smayda, T.J., Trainer, V.L. 2010. Dinoflagellate blooms in upwelling systems: Seeding, variability, and contrasts with diatoms bloom behavior. Prog. Oceanogr. 85, 92-107.

Stekoll, M.S., Clement, L.E., Shaw, D.G., 1980. Sublethal effects of chronic oil exposure on the intertidal clam *Macoma balthica*. Mar. Biol. 57, 51–60.

Stegeman, J.J., Schlezinger, J.J., Craddock, K.E., Tillitt, D.E. 2001. Cytochrome P4501A expression in midwater fishes; potential effects of chemical contaminants in remote oceanic zones. Environ Sci Technol. 35, 54–62

Stefanos Dailianis, S. 2010. Environmental impact of anthropogenic activities: the use of mussels as a reliable tool for monitoring marine pollution. In: Lauren, E., McGevin, L (Eds). Anatomy, Habitat and Environmental Impact. Nova Science Publishers, Inc. ISBN 978-1-61761-763-8

Siah, A., Pellerin, J., Amiard, J.C., Pelletier, E., Viglino, L., 2003. Delayed gametogenesis and progesterone levels in soft-shell clams (Mya arenaria) in relation to in situ contamination to organotins and heavy metals in the St.Lawrence River (Canada). Comp. Biochem. Physiol. C 135, 145–156.

Sobrino-Figueroa, A. S., Cáceres-Martínez, C. 2014. Evaluation of the effects of the metals Cd, Cr, Pb and their mixture on the filtration and oxygen consumption rates in *Catarina scallop*, juveniles *Argopecten ventricosus*. Laboratorio, 1, 2.

Sola, F., Masoni, A., Fossat, B., Porthé-Nibelle, J., Gentien, P., Bodennec, G., 1999. Toxicity of fatty acid 18:5n3 from Gymnodinium cf. mikimotoi: I. Morphological and biochemical aspects on Dicentrarchus labrax gills and intestine. J. Appl. Toxicol. 19, 279–84. doi:10.1002/(SICI)1099-1263(199907/08)19:4<279::AID-JAT579>3.0.CO;2-X

Solé M, Porte C, Albaigés, J. 1995. The use of biomarkers for assessing the effects of organic pollution in mussels. Sci Total Environ 159:147-153.

Solé, M., Buetm A., Ortiz, L. 2007. Bioaccumulation and biochemical responses in mussels exposed to the water-accommodated fraction of the Prestige fuel oil. Scientia Marina 71, 373-382.

Soriano, J.A., Viñas, L., Franco, M.A., González, J.J., Ortiz, L., Bayona, J.M., Albaigés, J., 2006. Spatial and temporal trends of petroleum hydrocarbons in wild mussels from the Galician coast (NW Spain) affected by the Prestige oil spill. Sci. Total Environ. 370, 80–90. doi:10.1016/j.scitotenv.2006.06.012

Soudant, P., Marty, Y., Moal, J., Samain, J.F., 1995. Separation of major polar lipids in *Pecten maximus* by high-performance liquid chromatography and subsequent determination of their fatty acids using gas chromatography. J. Chromatogr. B 673, 15–26

Stegeman, J.J., Lech, J.J., 1991. Cytochrome P-450 monooxygenase systems in aquatic species: carcinogen metabolism and biomarkers for carcinogen and pollutant exposure. Environ. Health. Perspect. 90, 101–109.

Strohmeier, T., Strand, Ø., Alunno-Bruscia, M., Duinker, A., Cranford, P., 2012. Variability in particle retention efficiency by the mussel *Mytilus edulis*. J. Exp. Mar. Biol. Ecol. 412, 96-102.

Suárez, P., Ruiz, Y., Alonso, A., San Juan, F., 2013. Organochlorine compounds in mussels cultured in the Ría of Vigo: Bioaccumulation and origin. Chemosphere. 90, 7-19.

Sukhotin, A.A., Pörtner, H.O., 2001. Age-dependence of metabolism in mussels *Mytilus edulis* (L.) from the White Sea. J. Exp. Mar. Biol. Ecol. 257 (1), 53-72.

Sukhotin, A.A., Abele, D., Pörtner, H. O. 2002. Growth, metabolism and lipid peroxidation in *Mytilus edulis L.*: age and size effects. Mar. Ecol., Prog. Ser. 226, 223–234.

Sukhotin, A.A., Lajus, D.L., Lesin, P.A., 2003. Influence of age and size on pumping activity and stress resistance in the marine bivalve *Mytilus edulis* L. J. Exp. Mar. Biol. Ecol. 284 (1/2), 129-144.

Sukhotin, A.A., Flyachinskaya, L.P., 2009. Aging reduces reproductive sucess in mussels *Mytilus edulis*. Mec. Ageing Develop. 130, 754-761.

Sureda, A., Box, A., Tejada, S., Blanco, A., Caixach, J., Deudero, S., 2011. Biochemical responses of *Mytilus galloprovincialis* as biomarkers of acute environmental pollution caused by the Don Pedro oil spill (Eivissa Island, Spain). Aquat. Toxicol. 101, 540–9. doi:10.1016/j.aquatox.2010.12.011

Tankoua, O.F., Buffet, P.E., Amiard, J.C., Amiard-Triquet, C., Mouneyrac, C., Berthet, B., 2011. Potential influence of confoundind factors (size, salinity) on biomarkers in the

sentinel species *Scrobicularia plana* used in programmes monitoring estuarine quality. Envion. Sci. Pollut. Res. 18, 1253-1263.

Thain, J.E., Vethaak, D., Hylland, K., 2008. Contaminants in marine ecosystems: developing an integrated indicator framework using biological-effect techniques. ICES. J. Mar. Sci. 65, 1-7.

Thébault, H., Rodriguez y Baena, A.M., Andral, B., Barisic, D., Albaladejo, J.B., Bologa, A.S., Boudjenoun, R., Delfanti, R., Egorov, V.N., El Khoukhi, T., Florou, H., Kniewald, G., Noureddine, A., Patrascu, V., Pham, M.K., Scarpato, A., Stokozov, N. a., Topcuoglu, S., Warnau, M., 2008. 137Cs baseline levels in the Mediterranean and Black Sea: A cross-basin survey of the CIESM Mediterranean Mussel Watch programme. Mar. Pollut. Bull. 57, 801–806. doi:10.1016/j.marpolbul.2007.11.010

Thiagarajan R, Gopalakrishnan S, Thilagam H. 2006. Immunomodulation the marine green mussel *Perna viridis* exposed to sub-lethal concentrations of Cu and Hg. Arch Environ Contam Toxicol. 51(3):392-9.

Thomas RE, Brodersen C, Carls MG, Babcock M, Rice SD. 1999. Lack of physiological resposes to hydrocarbon accumulation by *Mytilus trossulus* 3-4 years chronic exposure to spilled Exxon Valdez crude oil in Prince William Sound. Comp Biochem Physiol. Part C, 122: 153-163.

Thompson, R.J., Bayne, B.L., 1972. Active metabolism associated with feeding in the mussel *Mytilus edulis* L. J. Exp. Mar. Biol. Ecol. 9, 111–124.

Thompson, R.J., Bayne, B.L. 1974. Some relationship between growth, metabolism and food in the mussel, *Mytilus edulis.* Mar. Biol. 27, 317-326.

Timbrell, J.A. 2009. Principles of biochemical toxicology. Informa Healthcare. New York.

Toro, B., Navarro, J.M., Palma-Fleming, H., 2003. Use of clearance rate in *Choromytilus chorus* (Bivalvia: Mytilidae) as a non-destructive biomarker of aquatic pollution. Rev. Chi. Hist. Nat. 76, 267-274.

Tsangaris C, Papathanassiou , Nicolaidou A. 2008. Biochemical biomarkers and overall health state of mussels *Mytilus galloprovincialis* exposed to nickel and chromium. Chemistry and Ecology 24 (5), 315–327

Tsangaris, C., Cotou, E., Papathanassiou, E., Nicolaidou, A., 2010. Assessment of contaminant impacts in a semi-enclosed estuary (Amvrakikos Gulf, NW Greece): bioenergetics and biochemical biomarkers in mussels. Environ. Monit. Assess. 161, 259-69.

Urrutia, M.B., Navarro, E., Iglesias, J.I.P., 2001. Preingestive selection processes in the cockle *Cerastoderma edule*: mucus production related to rejection of pseudofaeces . Mar. Ecol. Prog. Ser. 209, 177-187.

US EPA, United States Environmental Protection Agency, 2001. Methods for Collection, Storage and Manipulation of Sediments for Chemical and Toxicological Analyses: Technical Manual. EPA 823-B-01-002. Office of Water, Washington, DC.

US EPA (2003). United States Environmental Protection Agency National air quality and emissions trends report—2003 special studies edition. EPA/454/R-03/005. Research Triangle Park, NC. URL http://www.epa.gov/air/airtrends/aqtrnd03/ (Acceso 13/12/2014).

US EPA. 2003. Ambient aquatic life water quality criteria for tributyltin (TBT) – Final. Office of Science and Technology EPA822-R-03-031. Washington, D.C.

US EPA. 2011. Pesticide News Story: EPA Releases Report Containing Latest Estimates of Pesticide Use in the United States. http://epa.gov/oppfead1/cb/csb page/updates/2011/sales-usage06-07.html

US EPA (2014). United States Environmental Protection Agency, List of Priority Pollutants.. URL http://water.epa.gov/scitech/methods/cwa/pollutants.cfm (acceso 28.09.2014).

Valavanidis, A., Vlahogianni, T., Dassenakis, M., Scoullos, M., 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxicant environmental pollutants. Ecotoxicantol. Environ. Saf. 64, 178-189.

Valette-Silver, N.J., Riedel, G.F., Crecelius, E.A., Windom, H., Smith, R.G., Dolvin, S.S., 1999. Elevated arsenic concentrations in bivalves form the southeast of the USA. Mar. Environ. Res. 48, 311-333.

Vahl, O., 1973. Pumping and oxygen consumption rates of *Mytilus edulis* L. of different size. Ophelia 12, 45-52.

Van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57-149.

Van Hattum B, Pons MJC, Montañés JFC. Polycyclic aromatic hydrocarbons in freshwater isopods and field partitioning between abiotic phases. Arch Environ Contam Toxicol 1998; 35:257–67

Vargas-Albores F, Barracco MA. 2001. Mechanismos de defensa de los moluscos bivalvos, con énfasis en pectínidos. In: Maeda-Martínez AM, editor. Moluscos Pectínidos de Iberoamérica: Ciencia y Acuicultura. Mexico: Limusa; 2001, 127-46.

Velasco, L.A., Navarro, J.M., 2003. Energetic balance of infaunal (*Mulinia edulis* King) and epifaunal (*Mytilus chilensis* Hupe) bivalves in response to wide variations in concentration and quality of seston. J. Exp. Mar. Biol. Ecol. 296 (1), 79-92.

Viarengo, A., Canesi, L., Pertica, M., Poli, G., Moore, M.N., Orunesu, M., 1990. Heavy metal effects on lipid peroxidation in the tissues of *mytilus galloprovincialis* lam. Comp. Biochem. Physiol. Part C Comp. Pharmacol. 97, 37–42. doi:10.1016/0742-8413(90)90168-9

Viarengo, A., Canesi, L., Pertica, M., Poli, G., Moore, M.N., Orunesu, M., 1990. Heavy metal effects on lipid peroxidation in the tissues of *mytilus galloprovincialis lam.* Comp. Biochem. Physiol. Part C Comp. Pharmacol. 97, 37–42. doi:10.1016/0742-8413(90)90168-9

Viarengo, A., Canesi, L. 1991. Mussels as biological indicators of pollution. *Aquaculture*, *94*(2), 225-243.

Viarengo, A., Abele-Oeschger, D., Burlando, B., 1998. Effects of low temperature on prooxidant processes and antioxidant defence systems in marine organisms. In: Portner H-O, Playle RC, editors. Cold ocean physiology. Cambridge University Press, Cambridge, 213–235.

Viarengo, A., Canesi, L., Pertica, M., Livingstone, D.R., 1991. Seasonal variations in the antioxidant defece systems and lipid peroxidation of the digestive gland of mussels. Comp. Biochem. Physiol. C. 100, 187-190.

Vidal-Liñán, L., Bellas, J., Campillo, J., Beiras, R., 2010. Integrated use of antioxidant enzymes in mussels, *Mytilus galloprovincialis*, for monitoring pollution in highly productive coastal areas of Galicia (NW Spain). Chemosphere. 78, 265-272.

Viñas, L., Franco, A., Blanco, X., Bargiela, J., Soriano, J., Perez-Fernandez, B., & Gonzalez, J. J. 2012. Temporal and spatial changes of PAH concentrations in Mytilus galloprovincialis from Ria de Vigo (NW Spain). *Environ.Sci.Res. 19*(2), 529–539.

Vidal-Liñán, L., Bellas, J., 2013. Practical procedures for selected biomarkers in mussels, *Mytilus galloprovincialis*: implications for marine pollution monitoring. Sci. Total Environ. 461-462, 56–64. doi:10.1016/j.scitotenv.2013.04.079

Villalba, A., 1995 Gametogenic cycle of cultured mussel, *Mytilus galloprovincialis*, in the bays of Galicia (NW Spain). Aquaculture, 130, 269-277.

Viñas Diéguez, L., Franco Hernández, A., González Fernández, J.J., 2002. Distribution of polycyclic aromatic hydrocarbons in superficial sediments of the Vigo estuary, Spain, central axis and adjacent shelf. Polycyclic Aromatic Compounds. 22, 161-173.

Viñas L., Franco M.A., Soriano J.A., González J.J., Ortíz L., Bayona J.M., Albaigés J., 2009. Accumulation trends of petroleum hydrocarbonsin commercial shellfish from the Galician coast (NW Spain) affected by the Prestige oil spill. Chemosphere 75:534–541.

Viñas, L., Franco, M.A., Blanco-García, X., Bargiela, J., Pérez-Guerrero, B., González, J.J. 2010. PAHs en sedimentos superficiales de las rías gallegas. XV Seminario Ibérico de Química Marina. Vigo. España. 2010.

Vlahogianni, T., Valavanidis, A., 2007. Heavy-metal effects on lipid peroxidation and antioxidant enzyme activities in mussels *Mytilus galloprovincialis*. Chem. Ecol. 23 (5), 361-371.

Walker, C.H., Hopkin, S.P., Sibly, R.M., Peakall, D.B. 2000. Major classes of pollutants. In: Taylor and Francis (Eds). Principles of Toxicology. Second edition, London, UK. pp 309.

Walker, C.H., 2009. Major classes of pollutants. In: Taylor and Francis (Eds). Organic pollutants and ecotoxicological perspective. Boca Raton, FL. pp 377.

Wang, S., Hong, H., Wang, X., 2005. Bioenergetic responses in green lipped mussels (*Perna viridis*) as indicators of pollution stress in Xiamen coastal waters, China. Mar. Pollut. Bull. 51, 738–43. doi:10.1016/j.marpolbul.2005.02.024

Wang, W., Wong, R.C.K., 2003. Combined effects of food quantity and quality on Cd , Cr , and Zn assimilation to the green mussel , *Perna viridis* 290, 49–69. doi:10.1016/S0022-0981(03)00070-4

Wang, S., Hong, H., Wang, X., 2005. Bioenergetic responses in green lipped mussels *(Perna viridis*) as indicators of pollution stress in Xiamen coastal waters, China. Mar. Pollut. Bull. 51, 738–43. doi:10.1016/j.marpolbul.2005.02.024

Ward, J.E., Levinton, J.S., Shumway, S.E., 2003. Influence of diet on pre-ingestive particle processing in bivalves I: Transport velocities on the ctenidium. J. Exp. Mar. Biol. Ecol. 293(2), 129-149.

Ward, J.E., Shumway, S.E. 2004. Separating the grain from the chaff: particle selection in suspension and deposit-feeding bivalves. J. Exp. Mar. Biol. Ecol. 300, 83-130.

Weinstein, J.E., 1997. Fluoranthene-induced histological alterations in oysters, *Crassostrea virginica*: Seasonal field and laboratory studies. Mar. Environ. Res. 43, 201–218. doi:10.1016/0141-1136(96)00088-8

Whittle, M.A. and Gabbott, P.A. 1986. Glycogen synthethase in the sea mussel *Mytilus edulis (L.).* III. Regulation by glucose in a mantle tissue slice preparation. Comp. Biochem. Physiol., 83B, 209–214

Whyte J.N.C., Englar J.R., Carswell B.L., 1990. Biochemical composition and energy reserves in *Crassostrea gigas* exposed to different levels of nutrition. Aquaculture 90, 157–172.

Widdows, J. 1978. Combined effects of body size, food concentration and season on the physiology of *Mytilus edulis.* J Mar Bio Ass. 58: 109-124.

Widdows, J., 1985. Physiological measurements In: The effect of stress and pollution on marine animals. Ed: B.L Bayne et al., pp 3-44. New York. Praeger.

Widdows, J., Moore, S.L., Clarke, K.R., Donkin, P., 1983. Uptake, tissue distribution and elimination of [114- C] naphthalene in the mussel *Mytilus edulis*. Mar Biol 76, 109-114.

Widdows, J., Bayne, B.L., 1971. Temperature acclimation of *Mytilus edulis* with reference to its energy budget. J. Mar. Biol. Assoc. U. K. 51 827-835.

Widdows, J., Fieth, P., Worrall, C.M., 1979. Relationships between seston, available food and feeding activity in the common mussel *Mytilus edulis*. Mar. Biol. 50, 195-207.

Widdows, J., Donkin, P., Salkeld, P.N., Cleary, J.J., Lowe, D.M., Evans, S.V., Thomson, P.E., 1984. Relative importance of environmental factors in determining physiological differences between two population of mussels (*Mytilus edulis*). Mar Ecol Prog Ser 17, 33-47.

Widdows, J., Johnson, D., 1988. Physiological energetics of *Mytilus edulis*: scope for growth. Mar. Ecol. Prog. Ser. 46,113–21.

Widdows, J., Donkin, P., 1989. The application of combined tissue residue chemistry and physiological measurements of mussel (*Mytilus edulis*) for the assessment of environmental pollution. Hydrobiologia. 188-189(1), 455–461.

Widdows, J., Hawkins, A.J.S., 1989. Partitioning of rate of heat dissipation by *Mytilus edulis* into maintenance, feeding, and growth components. Physiol. Zool. 62 (3), 764–784.

Widdows , J., Donkin, P., 1991. Role of physiological energetic in ecotoxicology. Comp. Biochem. Physiol. C. 100, 69-75.

Widdows, J., Nasci, C., & Fossato, V. U. 1997. Effects of pollution on the scope for growth of mussels (*Mytilus galloprovincialis*) from the Venice Lagoon, Italy. Mar.Environ. Res.43(1), 69-79.

Widdows, J., Donkin, P., Staff, F.J., Matthiessen, P., Law, R.J., Allen, Y.T., 2002. Measurement of stress effects (scope for growth) and contaminant levels in mussels (*Mytilus edulis*) collected from the Irish Sea. Mar. Environ. Res. 53, 327-356.

Widdows, J., Staff, F., 2006. Biological effects of contaminants: measurement of scope for growth in mussels. ICES TIMES Tecniques In Marine Environmental Sciences. 1-40.

Widdows, J., Bakke, T., Bayne, B.L., Donkin, P., Livingston, D.R., Lowe, D.M., Moore, M.N., Evans, S.V., Moore, S.L., 1982. Response of *Mytilus edulis* L. on exposure to the wateraccommodation fraction of North Sea oil. Mar. Biol. 67, 15–31

Widdows, J., Donkin, P., Salkeld, P.N., Cleary, J.J., Lowe, D.M., Evans, S.Y. & Thomson, P., 1984. Relative importance of environmental factors in determining physiological differences between two populations of mussels (*Mytilus edulis*). Mar.Eco/.Prog.Ser. 17 : 33-47.

Widdows J., Donkin P., Salkeld P.N., Evans S.V., 1987. Measurement of scope for growth and tissue hydrocarbon concentrations of mussels (*Mytilus edulis)* at sites in the vicinity of Sullom Voe: a case study. In: Kuipers J, Van den Brink WJ (eds) Fate and effects of oil in marine ecosystems. Nijhoff, Dordrecht, p 269–277

Widdows, J., Donkin, P., Brinsley, M.D., Evans, S.V., Salkeld, P.N., Franklin, A., Law, R.J., Waldock, M.J., 1995. Scope for growth and contaminant levels in North Sea mussels *Mytilus edulis*. Mar. Ecol. Prog. Ser. 127, 131–148.

Winston, G.W., Di Giulio, R.T., 1991. Prooxidant and antioxidant mechanisms in aquatic organisms. Aquat. Toxicol. 19, 137–161.

Winston, G.W., Di Giulio, R.T., 1991. Prooxidant and antioxidant mechanism in aquatic organism. Aquat. Toxicol. 24, 143–152.

Whiteley, C.G., Lee, D-J. 2006. Enzyme technology and bioremediation. Enzyme Microbiol. Technol. 38: 291-316.

Winter ,J.E., 1978. A review on the knowledge of suspension-feeding in lamelli-branchiate bivalves, with special reference to artificial aquaculture systems. Aquaculture. 13, 1-33.

Winberg, G.C.. 1956. Rate of metabolism and food requirements off fishes. Fish. Res. Board GUI. Truml. Ser.Vol. 194. pp. l-253.

Wootton, E.C., Dyrynda, E.A., Ratcliffe, N.A. Bivalve immunity: comparisons between the marine mussel (*Mytilus edulis*), the edible cockle (*Cerastoderma edule)* and the razor shell (*Ensis siliqua*), Fish Shellfish Immunol. 15, 195-210.

World Register Of Marine Species (WORMS). http://www.marinespecies.org

Wong, W.H., Cheung, S.G., 2001. Feeding rates and scope for growth of green mussels, *Perna Viridis* L . and their relationship with food availability in Kat O , Hong Kong 123–137.

Yap, C.K., Ismail, A., Tan, S.G., 2003. Effects of total soft tissue and shell thickness on the accumulation of heavy metals (Cd, Cu, Pb, and Zn) in the green-lipped mussel *Perna viridis*. Russ. J. Mar. Biol. 29(5), 323-7.

Yahel, G., Marie, D., Beninger, P. G., Eckstein, S., Genin, A. 2009. In situ evidence for pre–capture qualitative selection in the tropical bivalve *Lithophaga simplex*. Aquat. Biol. 6, 235-246.

Yao, C.L., Somero, G.N., 2013. Thermal stress and cellular signaling processes in hemocytes of native (*Mytilus californianus*) and invasive (*M. galloprovincialis*) mussels: Cell cycle regulation and DNA repair. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 165, 159–168. doi:10.1016/j.cbpa.2013.02.024

Yin, H., Xu, L., Porter, N. A. 2011. Free radical lipid peroxidation: mechanisms and analysis. Chem. Review. 111. 10, 5944-5972.

Zeng, Y., Jiang, W., Liu, Y., Wu, P., Zhao, J., Jiang, J., Kuang, S., Tang, L., Tang, W., Zhang, Y., Zhou, X., Feng, L. 2015. Dietary alpha-linolenic acid/linoleic acid ratios modulate intestinal immunity, tight junctions, antioxidant status and mRNA levels of NF-κB p65, MLCK and Nrf2 in juvenile grass carp (*Ctenopharyngodon idella).* Fish. Shellfish. Immun. In Press.

Zentz F, Hellio C, Valla A, De La Broise D, Bremer G, Labia R. 2002. Antifouling activities of N-substituted imides: antimicrobial activities and inhibition of *Mytilus edulis* phenoloxidase. Mar Biotechnol (NY). 4(4):431-40.

Zilberberg, C., Sereno, D., Lôbo-hajdu, G., 2011. Effect of Mussel' s Gender and Size on a Stress Response Biomarker 317–320. doi:10.1007/s11270-010-0589-4

Zippay, M.L., Helmuth, B. 2012. Effects of temperature change on mussel, *Mytilus.* Int. Zoo. 7, 312–327. doi: 10.1111/j.1749-4877.2012.00310.x

Zhong H., Kraemer, L., Evans, D., 2013. Influence of body size on Cu bioaccumulation in zebra mussels Dreissena polymorpha exposed to different sources of particle-associated Cu. J, Hazard, Mater, 261, 746-752.

Zuykov, M., Pelletier, E., Harper, D., 2013. Bivalve mollusks in metal pollution studies: From bioaccumulation to biomonitoring. Chemosphere. 93, 201-208.