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## Influence of mussel biological variability on pollution biomarkers



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## 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 the whole tissues of mussels.

A great geographical variability was observed for the biological variables, which was mainly linked to the differences in food availability along the studied region. An inverse relationship between antioxidant enzymes and the nutritional status of the organism was evidenced, whereas LPO was positively related to nutritional status 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.

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## 1. Introduction

Environmental monitoring programs have been traditionally designed for quantifying the concentration of chemical pollutants in different marine matrices: water, sediment and biota. These coastal pollution monitoring programs have included the analysis of biological effects caused in sentinel species (bioindicators) by the exposure to chemical pollution, in order to obtain combined chemical and biological data, to assess marine environmental quality (Bellás et al., 2014; Thain et al., 2008). Mussels are among the most commonly-used sentinel organisms in pollution studies due to its sedentary nature, its wide-spread geographical distribution, its great capacity for accumulating contaminants and its easy sampling (Kimbrough et al., 2008; Nakata et al., 2012; Sericano et al., 2014; Thébault et al., 2008; Widdows et al., 2002). Mussel biological responses (biomarkers) have been proposed as sensitive “early warning” tools to assess the environmental quality of coastal areas (Cajaraville et al., 2000; Lam, 2009) and some of

them have been incorporated by different international pollution monitoring programs (OSPAR, 2012a; ICES, 2013). Thus, biomarkers are 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 (Lam and Gray, 2003).

Pollutant-mediated generation of reactive oxygen species (ROS) is likely to induce antioxidant defense mechanisms in exposed organisms to prevent oxidative damage to cellular macromolecules (Livingstone, 2001). The measurement of the activity of antioxidant enzymes in mussels has been widely used as biomarkers of exposure to environmental pollutants (Lam, 2009). For the present study, we selected some exposure biomarkers (Catalase – CAT, Glutathione Reductase – GR and Glutathione S-transferase – GST activities) and an effect biomarker (Lipid peroxidation – LPO), which have been used to reveal the exposure to metals and a wide range of organic compounds in the environment (Campillo et al., 2013; Fernández et al., 2010a, 2010b, 2012; Fitzpatrick et al., 1997; Funes et al., 2006; Lee et al., 1988; Regoli, 1998; Vidal-Liñán et al., 2010). CAT is an extremely active catalyst for reduction of H<sub>2</sub>O<sub>2</sub> to H<sub>2</sub>O at high levels of H<sub>2</sub>O<sub>2</sub>, but at low levels it modulates

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the detoxification of other substances as phenols and alcohols through reactions coupled to the H<sub>2</sub>O<sub>2</sub> reduction. Although the GR does not play a direct role in the elimination of oxygen radicals, it can be regarded as an essential antioxidant enzyme since it reduces oxidized glutathione (GSSG) and maintains the GSSG/GSH balance, which is essential for cellular homeostasis and the operation of other enzymes (Regoli and Giuliani, 2013). GST is a Phase II detoxification enzyme involved in the conjugation and detoxification of organic compounds, and also plays a protective role against oxidative stress by catalyzing a selenium-dependent glutathione peroxidase (Prohaska, 1980; reviewed by Sheehan et al., 2001). Finally, 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; Regoli, 1998).

Regarding physiological biomarkers, the measurement of the scope for growth (SFG) has also been used as a measure of stress effects caused by pollutants in marine organisms (Albentosa et al., 2012a; Bellas et al., 2014; Halldórsson et al., 2005; Tsangaris et al., 2010; Widdows et al., 2002). SFG is a technique involving the calculation of the energy available for growth under standardized laboratory conditions. In short, it consists of evaluating the energy acquired by an organism after absorbing the food it has ingested, and that lost in the respiratory and excretory processes, being the difference the energy the organism has available for production (growth and reproduction) (Widdows and Johnson, 1988; Widdows and Donkin, 1989, 1991).

In general, biological responses used as biomarkers play a primary role in the normal homeostasis of the organism, and therefore, can also be influenced by natural biological and environmental cycles (Nahrgang et al., 2010). In fact, it has been previously highlighted that some confounding factors can alter biomarker responses to pollution (Coulaud et al., 2011; Lam, 2009). For instance, it has been reported that some enzymatic activities associated to biomarker responses change due to the natural physiological or reproductive cycles (Borkovic et al., 2005; Sheehan and Power, 1999), differences in food availability (Martínez-Álvarez et al., 2005) or water temperature (Viarengo et al., 1998), which may be misinterpreted as an exposure effect. These problems are magnified in large scale monitoring programs, as the present study, where organisms are subjected to a wide range of environmental conditions. Previous studies carried out within our group (Albentosa et al., 2012a; Bellas et al., 2014) have revealed that physiological and biochemical biomarkers seems to be more affected by mussel biological parameters than by chemical pollution. To validate the use of biomarkers in marine pollution studies it is indispensable to understand the importance of environmental variables on the studied biological responses (Tankoua et al., 2011).

The present study was carried out during the 2012 mussel Atlantic survey of the Spanish Marine Pollution monitoring programme, SMP, which is included within the framework of the Joint Assessment and Monitoring Programme (JAMP, OSPAR Commission). 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 Contaminants in Biota (OSPAR Commission, 2010a) and covers more than 2500 km of the N-NW Spanish coastline, located in the OSPAR Region IV. The SMP in this area consists of 40 sampling sites, from which 23 sites are monitored yearly. The sampling area includes two different oceanographic regions: the Atlantic and the Cantabrian coast 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 the upwellings 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 also 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).

The main objectives of the present study were to identify and characterize quantitative and qualitative biological variables (corporal and shell indices, gonadal development and reserves composition) that may affect the mussels' response to pollutant exposure in the populations of the SMP, and to establish the relationships between those biological variables and biomarkers responses, on the one hand, and between pollutants bioaccumulation and biomarkers on the other. The final objective of the study was to describe to what extent the variation in biomarkers responses can be explained by biological variables and by pollution.

## 2. Material and methods

### 2.1. Specimen sampling

Mussels ( $41.63 \pm 1.06$  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 considered for the spatial distribution studies of the SMP, since they cover a wide range of environmental scenarios (pollution sources, industrial areas, cities, upwelling, etc.). Table 1 shows the

**Table 1**

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
<i>Atlantic coast</i>				
Oia	2	41°58.212	08°53.199	5 H
Samil	3	42°13.177	08°46.604	2 H
Cabo Home	5	42°15.007	08°52.333	5 H
Pontevedra Raxó	7	42°24.172	08°44.968	2 H
Chazo	8	42°36.374	08°51.761	2 H
Punta Insua	12	42°46.423	09°07.550	5 H
Corme	14	43°14.569	08°56.642	3 H
A Coruña TC	16	43°22.178	08°23.160	5 H
Ferrol Palma	19	43°27.770	08°16.191	1 H
Cedeira	21	43°38.638	08°05.024	2 H
Espasante	22	43°43.346	07°48.302	4 H
<i>Cantabrian coast</i>				
Ribadeo	24	43°31.570	07°01.032	1 O
Luarca	26	43°32.872	06°32.409	3 O
Avilés	28	43°34.759	05°58.180	4 O
Gijón	29	43°34.174	05°43.329	5 O
Ribadesella	30	43°28.051	05°03.742	2 O
SV Barquera	31	43°22.885	04°23.803	1 O
Suances	32	43°26.338	04°02.621	3 O
Santander Pantalán	33	43°25.937	03°47.476	1 O
Santander Pedreña	34	43°26.929	03°45.173	1 O
Castro Urdiales	36	43°21.868	03°11.663	3 O
Bilbao Azcorri	37	43°22.917	03°00.885	5 O
Hondarribia	41	43°22.119	01°47.474	1 O

location of sampling sites and their environmental characteristics. 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 which primary production and upwelling event intensity decrease.

Fifty mussels were intended for chemical analyzes and thirty mussels were used for biological and biomarkers measurements. Mussels for chemical analysis were pooled (3 pools of 50 mussels per group) and soft tissues were separated from shells and triturated with Ultra-turrax. Mussels for biomarkers were placed in filtered seawater (1 µm) under controlled temperature (15 °C) and feeding conditions (microalgae *Isochrysis galbana*, 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. Samples for biomarkers and biological measurements were taken the day after the mussels arrived at the laboratory as recommended by Widdows and Staff (2006).

## 2.2. Biological measurements

### 2.2.1. Biometric parameters

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), which 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 aluminum foils whose weights were directly related to the weight of a rectangular aluminum 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 °C for 24 h to obtain individual dry tissue mass. General biological indices were calculated as follows:

Gill Index ( $GI$ ) = (gills dry weight/total meat dry weight) × 100  
 Gonadosomatic Index ( $GSI$ ) = (mantle dry weight/total meat dry weight) × 100  
 Hepatosomatic Index ( $HI$ ) = (digestive gland weight/total meat dry weight) × 100  
 Mussel condition was evaluated through two substantially different condition indices ( $CI$ ) according to Crosby and Gale (1990): Shell Condition Index ( $CI_{shell}$ ) = (total meat dry weight/shell dry weight) × 100  
 Volumetric Condition Index ( $CI_{vol}$ ) = (total meat dry weight/mussel volume) × 100  
 Mussel volume, expressed in ml, and considered as related to internal mussel cavity, was calculated from shell dimensions as follows =  $4/3 \times \pi \times (L/2) \times (H/2) \times (W/2)$ .

### 2.2.2. Biochemical composition

Mussel biochemical components were quantified in three pooled samples of five mussels per group. 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 soft tissues (mg/g AFDW). Carbohydrates were also expressed in absolute values for a mussel of a standard size of 8 ml (mussel

volumen) in order to avoid confusion due to relative percentages.

### 2.2.3. Gonadal development

Gonadal development was established for 15 mussels per site. For this, 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. Two additional indices were considered in this study, the sexual maturity index (SMI, Siah et al., 2003) that was the average gonadal development stage, and the total reproductive potential (TRP), calculated from  $TRP = (SMI \times GSI)$ .

## 2.3. Chemical analysis

### 2.3.1. Metals

Samples for metals analyzes 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 °C for 10 min and then at 180 °C 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<sub>2</sub> as reducing agent). Detection limits were 0.003, 0.005, 0.050, 0.50, 0.30, 0.30 mg/kg dry weight (mg/kg dw) for Hg, Cd, Pb, Cu, Zn and As, respectively.

### 2.3.2. Polycyclic aromatic hydrocarbons

PAH in mussel samples were Soxhlet extracted with a 3:1 hexane:acetone mixture and analyzed by high performance liquid chromatography (HPLC) as described elsewhere (Soriano et al., 2006). In summary, samples to be analyzed 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. The LOD was in the range of 0.1 (phenanthrene) to 0.4 µg/kg dw (indeno[1,2,3-cd]pyrene).

### 2.3.3. Polybrominated diphenyl ethers

For the analysis of PBDE congeners 28, 47, 66, 85, 99, 100, 153, 154, 183 homogenized 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 mass selective detector with

quadrupole analyzer 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 (octachloronaphthalene) the ion ( $m/z$ ) was 403.80. Methane was used as reagent gas. The LOD was 0.001  $\mu\text{g}/\text{kg}$  ww.

#### 2.3.4. Polychlorinated biphenyls and organochlorinated pesticides

The determination of the seven PCBs recommended by ICES ( $\Sigma_7$ PCBs, congeners 28, 52, 101, 118, 138, 153, 180), hexachlorocyclohexanes HCHs (sum  $\alpha$ -HCH and  $\gamma$ -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 determine 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 and He as carrier gas. Quantification was performed using multilevel calibration curves obtained by injection of standard solutions of seven different levels of concentration. The LOD was 0.01  $\mu\text{g}/\text{kg}$  ww.

#### 2.3.5. 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 ( $C_{crit}$ ) for each analyte (Bellas et al., 2011):  $CPI = \sum_i [\log(C_i/C_{crit_i})]$ . A principal component analysis (PCA) was carried out with the autocaled 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 considered 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.

#### 2.4. Biochemical biomarkers

Antioxidant biomarkers were measured in digestive glands from 10 mussels from each site which were pooled into groups of two ( $n=5$ ). 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. After sequential centrifugations at 10,000g for 20 min and 36,000g for 60 min, the resulting microsomal pellet (microsomal fraction) 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 microsomal fractions for LPO analysis.

CAT was measured according to Claiborne (1985) by the decrease in absorbance at 240 nm by  $\text{H}_2\text{O}_2$  consumption, and expressed as  $\mu\text{mol}$  of  $\text{H}_2\text{O}_2$  degraded  $\text{min}^{-1} \text{mg}^{-1}$  protein. GRx activity was measured according to Ramos-Martínez et al. (1983) by following the decrease of the absorbance at 340 nm due to the oxidation of NADPH and expressed as  $\text{nmol}$  of NADPH oxidized  $\text{min}^{-1} \text{mg}^{-1}$  protein. 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. LPO was quantified on gland microsomal fractions as thiobarbituric acid reactive substances (TBARS), following Buege and Aust (1978). Protein concentrations in both fractions (microsomal and cytosolic) were measured according to Lowry et al. (1951) by using bovine serum albumin as standard. All the enzymatic activities were expressed as  $\text{nmol}/\text{min}/\text{mg}$  protein.

#### 2.5. Scope for growth test

Physiological measurements were taken after the 24 h acclimatization period according to the procedures described by ICES for the use of mussel 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 °C and 0.55  $\text{mg}/\text{l}^{-1}$  organic matter obtain from *I. galbana*, clone T-ISO, and 0.24  $\text{mg}/\text{l}^{-1}$  inorganic matter obtain from previously ashed marine sediment). Particulate concentration was measured with a Coulter Multisizer III fitted with a 100  $\mu\text{m}$  orifice diameter tube.

Mussel clearance rate (CR) was measured according to the equation  $CR = f(C_i - C_o)/C_i$  (Hildreth and Crisp, 1976), where  $f$  is the flow of water expressed in  $\text{l h}^{-1}$ ,  $C_i$  is the inlet concentration and  $C_o$  is the outlet concentration, both expressed in particulate volume units,  $\text{mm}^3/\text{l}$ . Ingestion rate (IR,  $\text{mg}/\text{h}$ ) was estimated as the product of CR and the particulate organic matter (POM,  $\text{mg}/\text{l}$ ). POM was determined as the difference between total particulate matter (TPM) and particulate inorganic matter (PIM). TPM ( $\text{mg}/\text{l}$ ) was determined as the dry weight (24 h at 100 °C) of the suspension filtered and PIM was given as the weight remaining after ignition (1 h at 450 °C). AE was calculated from the percentage of POM in the food and in the feces according to Conover's ratio (1966),  $AE = ((F - E)/([1 - (E)F]) \times 100$ , where  $F$  is the food percentage of OM and  $E$  is the feces percentage of OM. Absorption rate (AR,  $\text{mg}/\text{h}$ ) was obtained by multiplying the ingestion rate by the absorption efficiency ( $AR = IR \times AE$ ). RR was obtained according to the equation  $RR = [(O_{2bl} - O_{2exp})/t] \times V$ , where  $O_{2bl}$  and  $O_{2exp}$  are the oxygen concentrations in the blank and experimental respirometers, respectively, expressed in  $\text{mg O}_2 \text{l}^{-1}$ ;  $t$  is the time, expressed in h; and  $V$  is the volume, expressed in l.

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. Respiration rates were weight-standardized for a 1 g dw specimen using the allometric exponent  $b=0.75$  (Vahl, 1973).

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 POM obtained from Widdows and Johnson (1988); 1 mg oxygen is equivalent to 0.6998 ml oxygen (Ansell, 1973); and 1 ml oxygen is equivalent to 20.33 J (Widdows and Johnson, 1988). Scope for growth (SFG) was calculated from the energy balance equation according to the expression  $SFG = (IR \times AE) - R$ , where IR is the consumption of the energy available in the diet, AE is the absorption efficiency and  $R$  is 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).

#### 2.6. Statistical analysis

Statistical analysis was performed using STATGRAPHICS centurion XV.II. When the requirements of normality (standardized

**Table 2**  
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, *Cl<sub>shell</sub>*: Shell condition Index, *Cl<sub>voi</sub>*: Volumetric condition index, *GI*: Gill Index, *GSI*: Gonadosomatic Index, *HI*: Hepatosomatic Index, *TRP*: total reproductive potential, *SMI*: sexual maturity index and *CH*: Carbohydrates for a standard individual of 8 ml of volume of internal cavity.

No	Sampling site	Biometric measurements											Biochemical measurements				
		<i>L/H</i>	<i>L/W</i>	<i>H/W</i>	<i>ST</i> mg/cm <sup>2</sup>	<i>Cl<sub>shell</sub></i>	<i>Cl<sub>voi</sub></i>	<i>GI</i>	<i>GSI</i>	<i>HI</i>	<i>RI</i>	<i>TRP</i>	<i>SMI</i>	Proteins mg/g organic matter	Carbohydrates	Lipids	CH mg/ind
<i>Atlantic coast</i>																	
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
<i>Cantabrian coast</i>																	
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

skewness and standardized kurtosis) and homogeneity of variances (Levene's test) were met, a Student's test ( $p < 0.05$ ) was performed to establish differences of biological variables between coasts. The relationships between variables were explored by means of Pearson's correlation analysis ( $p < 0.05$ ).

PCA was used to calculate the CPI index. 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  $\sigma$  is the standard deviation of the data. PCA analyzes were carried out in order to obtain the most relevant chemical variables in the CPI data set. The Mantel's test (Mantel and Valand, 1970) was performed in order to obtain an overall view of the relationship between biomarkers responses with biological and pollution parameters. 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).

### 3. Results

#### 3.1. Biological variables

Table 2 shows mussels 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 the wave exposure, according to the semi-qualitative classification shown in Table 1. 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<sup>2</sup> 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 due basically to 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 status (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 Fig. 1 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. Other sources of biological variability were related to main biochemical components of mussel tissues (Table 2). In general, the condition index was positively related to the amount of carbohydrates in mussels' tissues ( $r = 0.751$ ,  $p < 0.001$ ). Absolute reserves values ranged from almost 140 mg of carbohydrates per individual in site 7, to only 50.9 mg/ind in site 32. 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 ( $r = 0.647$ ,  $p < 0.001$ ).

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 examine differences between areas (Table 3). In general, Atlantic mussels exhibited a significant 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 (sites 30 or 37) 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 site 7, 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.

#### 3.2. Pollution

Table 4 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, and the most polluted sites were 30, 31 and 32; while Pb ranged from 0.662 to 33.4 mg/kg dw, and the most

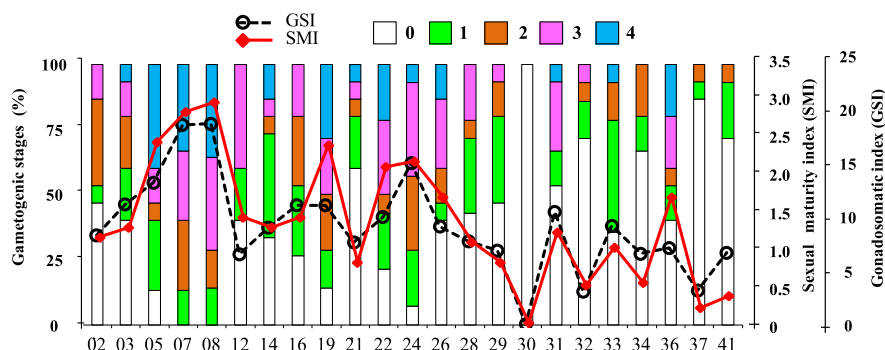


Fig. 1. Gametogenesis stages, variation of gonadosomatic index (GSI) and sexual maturity index (SMI) of mussels (*Mytilus galloprovincialis*), from the 23 sampling sites along the two geographical areas (Atlantic and Cantabrian coasts) collected in November of 2012.

**Table 3**  
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 maturity index, IND: percentage of indeterminate mussels, TRP: Total reproductive potential, CH/Lip: relationship carbohydrates–lipids and CH: Carbohydrates for a standard individual of 8 ml volume of internal cavity. Min: minimum value, Max: maximum value, R: range, SD: standard deviation, CV: variance coefficient and n: number of sites. Values in bold are statistically significant at  $p=0.05$  level.

Variable	Atlantic coast							Cantabrian coast							Atl. vs Cant.	
	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	<b>0.001</b>
$CI_{Vol}$	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	<b>0.021</b>
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	<b>0.002</b>
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	<b>0.006</b>
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	<b>0.028</b>
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	<b>0.019</b>
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	<b>0.000</b>
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	<b>0.000</b>

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, respectively, being higher also in the Cantabrian area. In contrast, Cd and As, which ranged from 0.388 and 4.07 to 2.90 and 12.8 mg/kg dw, respectively, showed higher values in the Atlantic area.

PAHs concentration ranged from 13.25 to 1015 µg/kg dw with highest concentrations found at sites 28, 29 and 33. PCBs ranged from 0.831 to 17.2 µg/kg ww and the highest values were found in sites 10, 19, 33 and 37. BDEs concentrations ranged from 0.057 to

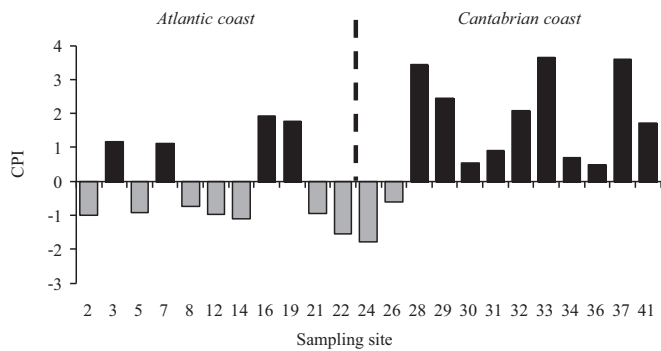
0.790 µg/kg ww, with the highest values detected in sites 16, 31 and 37. Organochlorinated pesticides, HCH, DDT and chlordanes ranged from 0, 0.077 and 0.022 to 0.078, 1.197 and 0.814, respectively, and the highest values were found in sites 28, 33 and 37 for HCH, 19, 33 and 41 for DDT, and 2, 12, and 31 for chlordanes.

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. Fig. 2 shows CPI values calculated for each sampling site, which takes positive values when pollutants exceed on average

**Table 4**  
Concentrations of pollutants in the soft tissues of mussels (*Mytilus galloprovincialis*) from the N–NW Spanish coast. Metals (mg/kg mussel dry weight),  $\Sigma_{12}$ PAHs: sum of 12 polycyclic aromatic hydrocarbons (µg/kg mussels dry weight),  $\Sigma_9$ BDEs: sum of 9 polybrominated diphenyl ethers (µg/kg mussels dry weight),  $\Sigma_7$ PCBs: sum of 7 polychlorinated biphenyls (µg/kg mussels dry weight),  $\Sigma$ DDTs: sum of p,p'-DDE, 4,4'-DDD and 4,4'-DDT (µg/kg mussels dry weight),  $\Sigma$ HCHs: sum of  $\alpha$ -HCH and  $\gamma$ -HCH,  $\Sigma$ Chlordanes: sum of trans-chlordane and cis-chlordane (µg/kg mussels dry weight), and CPI: Chemical Pollution Index.

No	Sampling site	Metals						$\Sigma_{12}$ PAHs	$\Sigma_9$ BDEs	Organochlorines				CPI
		Hg	Pb	Cd	Cu	Zn	As			$\Sigma_7$ PCB	$\Sigma$ DDTs	$\Sigma$ HCHs	$\Sigma$ chlordanes	
<i>Atlantic coast</i>														
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	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	Pontevedra 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
<i>Cantabrian coast</i>														
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	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	Santander Pantalán	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	Santander 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





**Fig. 2.** 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,  $\Sigma_9$ BDEs,  $\Sigma_7$ PCBs,  $\Sigma$ DDTs,  $\Sigma$ HCHs,  $\Sigma$ Chlordanes and B(a)p).

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 (Bellás et al., 2014): 'low pollution' when  $CPI \leq 0$ , 'moderate pollution' when  $0 < CPI \leq 1$  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  $\leq 0$ , 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.

### 3.2.1. Pollution vs biology

The relationship between biological parameters and chemical pollution were considered in this study (Table 5). According to the differences in biological variables showed above, subsequent analyzes were conducted in both regions separately. In Atlantic sites,  $Cl_{vol}$  was correlated positively with Cu, DDTs and PAHs. GSI showed positive correlations with PAHs. Regarding ST, negative correlations with some organic pollutants were observed, as with BDEs or PCBs.

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  $Cl_{vol}$  and CH.

### 3.3. Biochemical biomarkers

Biomarker results obtained in this study are shown in Table 6 and indicate a wide range of variation between sites. CAT values ranged from 35.5 to 104.1  $\mu\text{mol}/\text{min}/\text{mg}$  prot and presented the highest values in the stations 3, 5, 12, 19 and 37. GST and GR ranged from 48.0 to 142.1 and from 15.5 to 45.5  $\text{nmol}/\text{min}/\text{mg}$  prot 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  $\text{nmolMDA}/\text{mg}$  prot, with highest values measured in sites 21, 22, 24, 28 and 41. In general, antioxidant

**Table 5**

Pearson correlation coefficients among the different biological and chemical variables tested in the two different coasts (Atlantic and Cantabrian coasts). ST: Shell thickness,  $Cl_{vol}$ : Condition index, GI: Gill Index, GSI: Gonadosomatic Index, HI: Hepatosomatic Index, SMI: Sexual maturity index, CH: Carbohydrates per individual,  $\Sigma_{12}$ PAHs: sum of 12 polycyclic aromatic hydrocarbons,  $\Sigma_9$ BDEs: sum of 9 polybrominated diphenyl ethers,  $\Sigma_7$ PCBs: sum of 7 polychlorinated biphenyls,  $\Sigma$ DDTs: sum of p,p'-DDE, 4,4'-DDD and 4,4'-DDT,  $\Sigma$ HCHs: sum of  $\alpha$ -HCH and  $\gamma$ -HCH,  $\Sigma$ chlordanes: sum of trans-chlordane and cis-chlordane and CPI: Chemical pollution index. Values in bold are statistically significant at  $p=0.05$  level.

	CPI	Biological parameters						
		ST	$Cl_{vol}$	GI	GSI	HI	SMI	CH
<i>Atlantic coast</i>								
Cd	-0.424	0.438	-0.453	-0.0925	-0.457	0.149	-0.246	-0.241
Cu	0.483	-0.250	<b>0.695*</b>	-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	<b>0.785**</b>	-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
$\Sigma_9$ BDEs	<b>0.836**</b>	- <b>0.629*</b>	0.344	-0.2811	0.027	-0.206	-0.152	0.178
$\Sigma_7$ PCBs	<b>0.813**</b>	- <b>0.710*</b>	0.471	-0.1058	-0.014	-0.349	0.070	-0.223
$\Sigma$ HCHs	-0.096	0.184	-0.047	0.3537	0.061	0.354	0.107	-0.231
$\Sigma$ chlordanes	-0.216	0.406	-0.286	-0.0303	-0.411	0.357	-0.197	-0.338
$\Sigma$ DDTs	<b>0.738**</b>	-0.535	<b>0.678*</b>	0.0789	0.282	-0.079	0.386	-0.145
$\Sigma_{12}$ PAHs	<b>0.652*</b>	-0.345	<b>0.866***</b>	-0.1104	<b>0.603*</b>	0.236	0.422	0.301
CPI		-0.571	0.581	-0.1523	0.298	-0.019	0.155	0.259
<i>Cantabrian coast</i>								
Cd	<b>0.677*</b>	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	<b>-0.611*</b>	0.054	-0.487	<b>-0.576*</b>
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
$\Sigma_9$ BDEs	0.249	-0.022	-0.162	0.280	-0.120	-0.377	-0.251	0.042
$\Sigma_7$ PCBs	<b>0.676*</b>	-0.154	0.045	0.096	-0.152	-0.035	-0.360	0.281
$\Sigma$ HCHs	<b>0.713**</b>	0.133	-0.004	-0.101	0.069	-0.221	-0.032	0.085
$\Sigma$ chlordanes	-0.539	0.196	0.14	-0.223	0.335	0.120	0.386	0.090
$\Sigma$ DDTs	0.115	-0.329	0.305	-0.220	0.151	-0.202	-0.144	0.336
$\Sigma_{12}$ PAHs	<b>0.716**</b>	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

\* Correlations are significant at 0.05 level.

\*\* Correlations are significant at 0.01 level.

\*\*\* Correlations are significant at 0.001 level.

**Table 6**

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/ mg prot	GST nmol/min/mg prot	GR nmol/min/ mg prot	LPO nmolMDA/ mg prot
<i>Atlantic coast</i>					
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
<i>Cantabrian coast</i>					
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

activities were positively correlated between them and inversely correlated to LPO (data not shown), although these relationships were more evident in Cantabrian sites.

### 3.3.1. Biomarkers vs biology

Correlations between biological indices and biomarkers are shown in Table 7. In the Atlantic coast, GST showed a significant negative correlation with SMI and GR showed a negative correlation with GSI.

Biochemical biomarkers showed a similar pattern in the Cantabrian coast, where CAT was negatively correlated with GSI and SMI. Contrary to antioxidant activities, LPO showed strong positive correlations with Cl<sub>vol</sub>, GSI and CH, and also a negative correlation with GI.

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

### 3.3.2. Biomarkers vs pollution

Correlations between biomarkers and pollutants are shown in Table 7. Significant positive correlations were observed in the Atlantic coast between CAT and metals as As b. GR showed also positive correlations with Cd, Zn and also with chlordanes.

In the Cantabrian coast, CAT was positive correlated with Cu; GST was positively correlated with Cu and HCHs; GR showed a strong positive correlation with As ( $r=0.728$ ,  $p<0.01$ ) whereas LPO did not show any significant correlation with pollutants.

**Table 7**

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: Glutathione S-transferase (nmol/min/mg prot), GR: Glutathione Reductase (nmol/min/mg prot) and LPO: Lipid Peroxidation (nmolMDA/mg prot), ST: Shell thickness, Clvol: Gonadosomatic Index, HI: Hepatosomatic Index, SMI: Sexual maturity index, CH: Carbohydrates per individual, Σ12PAHs: sum of 12 polycyclic aromatic hydrocarbons, Σ9BDEs: sum of 9 polychlorinated biphenyls, 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. Values in bold are statistically significant at  $p=0.05$  level.

	Biological parameters													Chemical data												
	ST	Cl <sub>vol</sub>	GI	SMI	CH	CH	Cd	Cu	Hg	Pb	Zn	As	Σ <sub>9</sub> BDEs	Σ <sub>7</sub> PCB	ΣHCHs	Σchlordanes	ΣDDTs	Σ <sub>12</sub> PAHs	CPI							
<b>CAT</b>	-0.111	-0.083	0.266	0.187	0.055	0.130	0.177	-0.153	-0.031	0.542	0.309	<b>0.605*</b>	0.154	0.372	-0.163	0.298	0.360	-0.208	0.374							
<b>GST</b>	-0.044	-0.195	-0.424	-0.017	<b>-0.629*</b>	-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							
<b>GR</b>	-0.033	-0.576	0.197	-0.021	-0.395	-0.202	<b>0.613*</b>	-0.391	-0.518	0.387	<b>0.608*</b>	0.557	0.056	0.036	-0.114	<b>0.630*</b>	-0.214	-0.499	-0.102							
<b>LPO</b>	0.245	0.465	-0.286	-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							
<i>Atlantic coast</i>																										
<b>CAT</b>	-0.407	-0.490	0.411	-0.222	<b>-0.591*</b>	-0.168	0.217	<b>0.659*</b>	0.309	-0.211	-0.072	0.523	0.562	0.400	0.414	0.161	0.131	0.084	0.427							
<b>GST</b>	-0.298	-0.359	0.227	-0.562	-0.298	-0.298	0.058	<b>0.593*</b>	-0.158	-0.293	-0.161	0.554	0.153	0.261	<b>0.584*</b>	-0.239	0.081	0.383	0.435							
<b>GR</b>	-0.181	-0.452	0.359	-0.425	-0.389	-0.451	0.226	0.392	0.481	0.005	-0.224	<b>0.727**</b>	-0.075	-0.267	0.022	-0.265	-0.401	0.216	0.210							
<b>LPO</b>	0.297	<b>0.738**</b>	<b>-0.698*</b>	0.329	0.461	<b>0.625*</b>	-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							
<i>Cantabrian coast</i>																										

\*\*\*Correlations are significant at 0.001 level.

\* Correlations are significant at 0.05 level.

\*\* Correlations are significant at 0.01 level.

**Table 8**

Physiological parameters (mean and standard deviations) of mussels collected through the 23 sampling sites of the N-NW Spanish Coast under standardized laboratory conditions (15 °C, filtered seawater 1 µm, 0.55 mg/L of algal cells). CR<sub>L-st</sub>: Clearance rate (60 mm length), CR<sub>W-st</sub>: Clearance rate (1 g dry weight), AR: absorption rate, AE: absorption efficiency, RR: respiration rate and SFG: Scope for Growth.

No	Sampling site	Physiological parameters						
		CR <sub>L-st</sub> L/h	CR <sub>W-st</sub> L/h	IR J/g/h	AE %	AR J/g/h	RR J/g/h	SFG J/g/h
<i>Atlantic coast</i>								
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
<i>Cantabrian coast</i>								
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

### 3.4. Physiological biomarkers

Mean SFG values and their standard deviations are shown in Table 8, together with all the physiological rates used in the SFG estimation. SFG ranged from 7.0 to 31.0 J/g/h 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<sub>W-st</sub> ranged from 2.0 to 4.3 l/h, for a specimen of 1 g dry weight. When CRs were length-standardized (CR<sub>L-st</sub>) the range of CR values was lower (2.5–4.3 l/h 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<sup>-1</sup> h<sup>-1</sup>.

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.

#### 3.4.1. Biomarkers vs biology

In the Atlantic coast, there was not detected any relationship between physiological measurements and biological parameters (Table 9). On the contrary, highly significant relationships between physiological biomarkers and biological parameters were found in the Cantabrian coast. Independently of the standardization method used, CR was higher in mussels of lower condition, measured as CI<sub>vol</sub>, GSI or SMI, but this relationship was stronger for CR<sub>W-st</sub>. A positive correlation with GI was also evident. 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<sub>vol</sub>  $r=-0.683$ ,  $p < 0.05$ , 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.

#### 3.4.2. Biomarkers vs pollution

As well as with biochemical biomarkers, chemical pollution was associated with physiological biomarkers (Table 9). In the Atlantic coast, CR<sub>W-st</sub> did not show any correlation with pollution, however CR<sub>L-st</sub> was positively correlated with Hg. AE showed positive correlations with Pb. RR showed a positive correlation with Cu and a negative correlation with Zn. Finally, SFG showed positive correlations with metals as Pb or As.

In the Cantabrian coast, only As showed correlations with physiological biomarkers in this area. Positive correlations were found between As and AE or AR and as result, also with SFG.

## 4. Discussion

### 4.1. Biological variability

The applicability of biomarkers in marine pollution monitoring programs is conditioned by the capability of discriminating 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 status. In the present study, we have characterized the wide biological variability in the mussels populations used for the assessment of pollution along an

**Table 9**  
Pearson correlation coefficients among the physiological parameters considered and biological/chemical variables tested in the two different coasts (Atlantic and Cantabrian coasts), CR<sub>w-st</sub>: Clearance rate (1 g dry weight), CR<sub>l-st</sub>: Clearance rate (60 mm length), AR: absorption rate, AE: absorption efficiency, RR: respiration rate, SFC: scope for growth, ST: Shell thickness, Cl<sub>vol</sub>: Condition index, GI: Gill Index, GSI: Gonadosomatic Index, HI: Hepatosomatic Index, SMI: Sexual maturity index, Σ<sub>12</sub>PAHs: sum of 12 polycyclic aromatic hydrocarbons, Σ<sub>9</sub>BDEs: sum of 9 polybrominated diphenyl ethers, Σ<sub>7</sub>PCBs: sum of 7 polychlorinated biphenyls, 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. Values in bold are statistically significant at p=0.05 level.

Biological parameters		Chemical data																	
ST	Cl <sub>vol</sub>	GI	GSI	HI	SMI	Cd	Cu	Hg	Pb	Zn	As	Σ <sub>9</sub> BDEs	Σ <sub>7</sub> PCBs	ΣHCHs	Σchlordanes	ΣDDTs	Σ <sub>12</sub> PAHs	CPI	
<i>Atlantic coast</i>																			
CR <sub>w-st</sub>	-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 <sub>l-st</sub>	-0.001	-0.206	0.361	0.309	0.312	-0.086	0.118	<b>0.590*</b>	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	0.077	0.017	0.025	-0.121	0.454	<b>0.658*</b>	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	0.123	-0.013	0.165	-0.227	0.443	<b>0.734*</b>	0.498	<b>0.620*</b>	0.424	0.28	0.122	0.223	0.197	-0.05	0.577	
RR	0.303	0.486	0.138	0.374	0.248	-0.174	<b>0.654*</b>	0.022	-0.374	<b>0.633*</b>	-0.368	-0.180	0.029	0.303	0.107	0.257	0.391	-0.060	
SFC	-0.293	-0.134	0.187	0.032	-0.048	0.174	-0.299	0.397	<b>0.717*</b>	0.541	<b>0.613*</b>	0.408	0.249	0.067	0.186	0.141	-0.100	0.531	
<i>Cantabrian coast</i>																			
CR <sub>w-st</sub>	-0.423	<b>-0.894***</b>	<b>-0.845***</b>	-0.218	<b>-0.593*</b>	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 <sub>l-st</sub>	-0.205	<b>-0.758**</b>	<b>-0.603*</b>	-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	0.208	0.306	-0.028	-0.148	-0.066	<b>0.731**</b>	0.260	-0.010	0.437	-0.220	-0.290	0.356	0.321	
AR	-0.301	<b>-0.725**</b>	<b>-0.640*</b>	-0.384	-0.548	0.348	0.375	0.213	0.100	0.118	<b>0.726**</b>	0.384	0.119	0.332	-0.200	-0.370	0.148	0.448	
RR	<b>-0.725**</b>	-0.351	0.477	-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	
SFC	-0.225	<b>-0.683*</b>	<b>-0.667*</b>	-0.337	-0.489	0.379	0.351	0.176	0.120	0.162	<b>0.755**</b>	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.

extensive area included within the Spanish Marine Pollution monitoring programme (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 status. Mantle tissues serve to both accumulation of reserves and gonad development in *Mytilus* sp. (Gabbott and Peek, 1991). In this line, a general annual reproductive/reserves cycle has been described 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 markedly within the same population depending on latitude and on food availability (Gabbott, 1975), 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), the same as mussels from the area considered in 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) (Caceres-Martinez 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) distinguishes between “conservative species” which show one cycle of reserves storage in summer, gametogenesis in autumn-winter and spawning episodes in spring, from “opportunistic species” whose gametogenesis takes place whenever food availability conditions were optimal. *Mytilus* sp. behaves as “conservative species” in situations of low food availability and as “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 showed 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 a 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 gametogenesis stages (SMI, stages 3 or 4), indicated by the strong positive correlation between both variables (Fig. 1). This finding is in agreement with the “opportunistic strategy” of *Mytilus* sp. populations, according to the classification of Bayne (1976), in such a way that, if environmental conditions are favorable, mussels build up an 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; Suárez et al., 2013). Therefore, the variability of gonadal development in the study area, which covers more than 2500 km of coastline, is higher than the expected variability in one site throughout a year, i.e. all gametogenesis 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 up to 40% of the total organic matter. These values are included within the levels described for this season (autumn) in areas from the Galician Atlantic coastline, and also within the

levels registered in spring-summer where the 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 literature (de Zwaan and Zandee, 1972; Gabbott and Peek, 1991; Freites et al., 2003).

A clear geographical pattern was observed here both for the gonadal development (Fig. 1) and for the carbohydrates levels (Table 2). Atlantic populations show higher values of energetic reserves and also a highly differentiated gonadal development, in contrast to Cantabrian populations. Both, gonadal development and reserves levels, are closely linked to food availability (Bayne, 1976; Gabbott, 1976), suggesting that Atlantic habitats are more productive than 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 (Caceres-Martinez 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 the surface waters leading to an increase of primary productivity in these waters (Álvarez et al., 2011). Within the Atlantic coast, in the so called "Rías Baixas" (South Galicia) the upwelling phenomenon takes place with higher intensity (Gomez-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).

#### 4.2. Pollution

A clear difference in the levels of pollutants was observed between the two studied areas, as revealed by the CPI index (Fig. 2). In general, the Atlantic coast showed less pollution levels, although some stations (3, 7, 16 and 19 presented medium values of pollution due to the accumulation of industrial and urban activities, as reported elsewhere (Bellás et al., 2014 and references

therein). The most polluted sites (CPI > 2) are located in the Cantabrian area: sites 28 presented high values of Pb, PAHs and HCHs, site 29 showed high values of Hg and PAHs, site 32 showed high values of Hg, Pb and PCBs, site 33 presented high concentrations of PAHs, PCBs, and DDTs, and 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 (iron and steel industry, shipbuilding, chemical and pharmaceutical industry and wood processing) and port activities, which result in water quality degradation. Site 32, though, is a small fishing town, 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 (Irbien 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 Bellás 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 mussels tissues and pollution.

It is noteworthy the inverse relationship between the accumulation of certain organic pollutants such as PCBs 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, 2013). The effect of animal size on pollutant bioaccumulation, have been related to variations in physiological processes, as feeding, which depends of size and age (Zhong et al., 2013). On the other hand, this finding may be also related to the alteration produced by these contaminants on the normal process of calcification of the shell (Lionetto et al., 2012; Zuykov et al., 2013)

#### 4.3. Biochemical biomarkers

All the antioxidant activities measured were positively correlated between them and inversely related to lipid peroxidation due to the capacity of the antioxidant defense 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 et al., 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 pointed 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 status 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 the higher metabolic status of the animals in this season which is related to the gonad ripening and the higher food availability. However, we have found higher antioxidant activities in mussels with lower sexual maturity index. An alternative explanation for the higher antioxidant activities observed in the warmer months of the year 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 defense against oxidative stress as antioxidant enzymes (Pörtner, 2002). In this way, Borkovic et al. (2005) detected an increase of CAT activity in mussels from the Adriatic Sea collected in May, and suggest that this increase is linked to the increased metabolic activity related to the 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 due to a higher sensitivity to pollutants in the winter season (Borkovic et al., 2005), in the same way as described in present paper. In addition to 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 status were also described in limpets (Ansaldo et al., 2007) and in fishes (Martínez-Álvarez 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). 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 defense systems, as the antioxidant enzymes, could be activated. Moreover, an increase of the enzymes's activity responsible of the intermediary metabolism is an indication of the operation of the aerobic metabolism under condition of food restriction which has been previously described by Morales et al. (2004). For this reason, it has been postulated that animals living in fluctuating environments, as is the case of 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 defense systems by natural food limitation (Moore et al., 2006).

The present study also reports a positive correlation of the LPO with nutritional status (CI, GSI or CH) in mussels from the Cantabrian coast, suggesting that higher nutritional status may be associated with higher reproductive efforts and consequently, with higher metabolic costs, with their associated ROS generation (Bayne, 1976; Dahloff 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 enzymes activities. It is well known that the 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 analyzes conducted here, point to the metals as the main responsible of the observed effect on biochemical biomarkers, in particular Cu, Zn, Cd and As. The relationship between GR and metals could be attributed to a coordinate 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 Borkovic-Mitic 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 of enhancing 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 exerts their effects by blocking the hydrolysis of the neurotransmitter acetylcholine, which may be related to the generation of free radicals after metabolic activation with enzyme induction of P450 and resulting products may enter reversible oxidation (Lushchak, 2011). On the other hand, 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 (Bochetti and Regoli, 2006).

#### 4.4. 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 the different sites. This biological approach allows us to deepen understanding and clarify the 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 Crandford et al., 2011). However, changes in body mass due to seasonal changes in reserves or reproductive tissues are not related to gill size and this introduces standardization errors (Crandford 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 showed 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  $CR_{L-st}$  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 way, when food availability is high (Atlantic area) CR is not negatively affected by mussel condition. On the contrary, when food is less abundant (Cantabrian area) a clear negative effect of mussel condition is observed on CR.

Feeding behavior 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, 2002; 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".

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 and 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 variable 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 status 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, high AE values were observed in highpolluted sites, and no relationships between AE and biological parameters were identified.

Therefore an alternative hypothesis needs to be considered. Low AE was obtained when fecal 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). Feces 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 mussel's metabolism was higher in younger mussels. 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

**Table 10**

Mantel's test analysis for biochemical and physiological biomarkers with biological and chemical variables for the two coasts studied. Values in bold are statistically significant at  $p=0.05$  level.

Coast		Biochemical biomarkers vs		Physiological biomarkers vs	
		Biology	Pollution	Biology	Pollution
		Atlantic coast	<i>r</i>	0.003	−0.090
	<i>p</i> –value	<b>0.980</b>	<b>0.540</b>	<b>0.840</b>	<b>0.182</b>
Cantabrian coast	<i>r</i>	0.420	−0.155	0.600	−0.077
	<i>p</i> –value	<b>0.018</b>	<b>0.490</b>	<b>0.006</b>	<b>0.720</b>

condition on absorption rates described before. Relationships observed in the Atlantic mussels between some pollutants, as Pb or As, with SFG was contrary to expected (positive relationship). Since Pb levels in Atlantic mussels are low and significantly lower than in Cantabrian ones, an effect of this pollutant on SFG should be discarded. With regard to As, and as mentioned before, another variable which correlates with As levels might be responsible for the detected relationship.

#### 4.5. Comparative effects of biology and pollution on biomarkers responses

In order to obtain an overview of the relative influence of biological and pollution variables on both set of biomarkers, physiological and biochemical, a Mantel's test was performed (Mantel and Valand, 1970). 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 analyzes shown in Table 10. 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 biomarkers matrices.

Many non-pollution-related variables as the ones considered here (nutritional status, 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 et al., 2003). When a wide area is monitored as in the SMP, strong differences occur in the habitats where mussels are sampled, which determine mussel physiology and consequently those biological parameters used as pollution biomarkers. Among these habitat differences, food availability is one of the most important factors which affects general animal status due to its influence on gonadal development (Bayne, 1976; Barber and Blake, 1991), energy balance (Bayne 1973; Albentosa et al., 2012b) and nutrients composition (Navarro et al., 1989; Orban et al., 2010; 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.

## 5. Conclusions

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, and 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 large geographical variability was observed for the studied biological variables, including mussels' nutritional status, biochemical composition, gonadal development and age. This variability was mainly linked to the differences in food availability between both regions. Thus, although the requirements for mussel biomonitoring have been achieved: same size to guarantee the same age, and sampling at the same time of the year to ensure a similar reproductive stage, we found a great variability in both: age and reproductive stage, which may involve an important handicap for the development of biological monitoring programmes.

In general, antioxidant biomarkers in mussels presented higher values in mussels with poorer condition and with lower sexual maturity index, which indicates an inverse relationship between antioxidant enzymes and the nutritional status of the organism. In contrast, LPO was positively related to nutritional status 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. 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, although 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 present study, provides useful information to determine the physiological status 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 biomarkers responses. Both, biological and environmental parameters should be considered complementary to biomonitoring procedures. On the other hand, laboratory experiments where biological and pollution variables can be easily isolated, should be carried out with the purpose of determining the biomarker responses to contaminants over a wide range of environmental and intrinsic conditions.



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