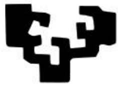

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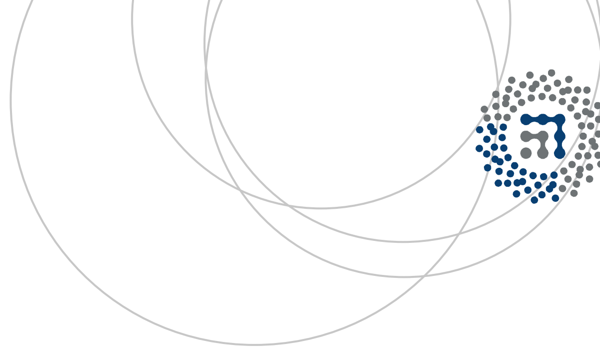

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Biochemical biomarkers in samples processed by different methods in Environmental Specimen Banks

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1. ABSTRACT

In the last decades the creation of new Environmental Specimen Banks (ESB) is increasing due to the necessity of knowing the effects of pollutants in both the environment and human populations. ESBs analyze and store samples in order to understand the effects of chemicals, emerging substances and the environmental changes in biota. For a correct analysis of the effect induced by these variables, there is a need to add biological endpoints, such as biomarkers, to the endpoints based on chemical approaches which have being used until now. It is essential to adapt ESB´s sampling strategies in order to enable scientists to apply new biological methods. The present study was performed to obtain biochemical endpoints from samples stored in the BBEBB (Biscay Bay Environmental Biospecimen Bank) of the Marine Station of Plentzia (PIE - UPV/EHU). The main objective of the present work was to study the variability caused in biochemical biomarkers by different processing methods in mussels (*Mytilus galloprovincialis*) from two localities (Plentzia and Arriluze) with different pollution history. It can be concluded that the selected biomarkers (glutathione S-transferase and acetylcholinesterase) can be accurately measured in samples stored for years in the ESBs. The results also allowed the discrimination of both sampling sites. However, in a further step, the threshold levels and baseline values should be characterized for a correct interpretation of the results in relation to the assessment of the ecosystem health status.

2. INTRODUCTION

Over the last twenty centuries, environment has been under the stress produced by increasing anthropogenic activities that cumulated over the last 100 years. An increasing number of pollutants, organic or inorganic, are suspected to affect the environment, but their toxicity is only known in a few cases. According to the European Inventory of Existing Commercial Substances, more than 100.000 chemical compounds are produced in the world every year and around 1.000 and 2.000 enter the worldwide market adding to those that already exist. Some of these emerging pollutants are being produced in large quantities and their properties, transformations, biological impact (short- and long-term) on biota and ecosystems (toxicity and ecotoxicity), global distribution, and final fate of most pollutants remains unknown or poorly known. Moreover, many compounds present low degradability and are therefore highly persistent and susceptible to be biomagnified throughout the trophic chains (Wu, 1999; Letcher et al., 2010).

Overall, the chemical analysis and detection capacity for many chemicals is still limited; thus, the risk arising from chemical exposure of the environment cannot be

adequately described. Being the watchtowers of environmental safety, the Environmental Specimen Banks (ESBs) provide a wide view on chemical contamination. They are the drivers for spatial and temporal trend analysis using archived samples, some of them stored under good quality control for more than decades. Even in the past ESBs provide valuable data mainly for regulators and industry, nowadays, scientists are integrating these results to improve the understanding of the fate and behavior of chemicals and their biological effects, both in the environment and human populations (Garmendia et al., 2015).

Environmental specimen banks (ESBs) are facilities that are dedicated to the systematic, long-term archival of biotic or abiotic environmental samples for monitoring and research. The history of environmental specimen banks goes back to the 1960s (Odsjö, 2006), when the first biobank was established in the Swedish Museum of Natural History, Stockholm and then in 1979 in the USA (Becker and Wise, 2006) and in Germany (BMU 2008). Since then, biobanking activity has progressed and several ESBs in the North, Central, and South of Europe have been launched. One of those banks is the BBEBB (Biscay Bay Environmental Biospecimen Bank) of the Marine Station of Plentzia (PIE - UPV/EHU) which was created in 2012. Today, environmental specimen banking is flourishing, not just in Europe and USA but also in Australia, China, and other Asian countries (Day et al., 2014). Twenty-five formal ESBs now exist in 14 countries (**Table 1**), with new facilities in other countries currently under development.

Table 1. List of formal environmental specimen banks (ESBs) that currently exist or are under development (Day et al., 2014).

Country	Name	Location
Canada	Canadian Wildlife Service Specimen Bank	National Wildlife Research Centre
Canada	National Aquatic Biological Specimen Bank and Database	Canada Centre for Inland Waters, Environment Canada
China	Yangtze Environmental Specimen Bank	Tongji University, Jiaxing
Denmark	Tissue and Data Bank for Greenland	National Environmental Research Institute
Denmark	Faroe Islands Environmental Specimen Bank	Environment Agency, Traðagøta, Faroe Islands
Finland	Paljakka Environmental Specimen Bank	Finnish Forest Research Institute
France	Observatoire de Recherche sur l'Environnement (ORQUE)	University of Pau
France	ANDRA Observatoire Perenne de l'Environnement (OPE)	University of Pau
France	Mytilothèque	French Research Institute for Exploitation of the Sea (Ifremer),
Germany	German Environmental Specimen Bank	Federal Environment Agency, Dessau-Roßsslau
Italy	Mediterranean Marine Mammal Tissue Bank	University of Padua
Italy	Antarctic Environmental Specimen Bank (BCAA)	Genoa
Japan	Environmental Specimen Bank for Global Monitoring (es-Bank)	Ehime University
Japan	Time Capsule for Environment and Endangered Wildlife	National Institute of Environmental Studies
Norway	Norwegian Environmental Specimen Bank	Oslo Centre for Interdisciplinary Environmental and Social Research
Spain	Biscay Bay Environmental Biospecimen Bank	University of the Basque Country, Plentzia
Spain	Environmental Specimen Bank of Galicia	University of Santiago De Compostela
South Africa	Biological Resource Bank	National Zoological Gardens
South Korea	National Environmental Specimen Bank	National Institute of Environmental Sciences, Seoul
South Korea	South Sea Research Institute (SSRI)	Geoje
Sweden	Environmental Specimen Bank	Swedish Museum of Natural History
UK	National Fish Tissue Archive	Centre for Ecology and Hydrology
USA	Marine Environmental Specimen Bank	National Institute of Standards and Technology Charleston, SC
USA	CDC and ASTDR Specimen Packaging, Inventory, and Repository	Centers for Disease Control and Prevention
USA	Alaska Frozen Tissue Collection	Museum of the North, University of Alaska, Fairbanks

Historically, the primary reason for environmental specimen banking was to provide researchers and ecologists with materials that could be used for analyzing temporal trends in exposure for previously unrecognized pollutants or for pollutants for which analytical techniques were inadequate at the time of collection. Twenty first century's ESBs will continue playing this important role in the exposure assessment of known chemicals and of emerging substances (Tanabe and Ramu, 2012). In order to achieve this objective the ESBs collect and process samples without altering their original composition, and preserve them in a stable environment over extremely long time-frames. This typically means processing samples under clean room conditions using carefully chosen materials and detailed protocols, storing specimens under cryogenic temperatures, and using well-designed sample inventory and tracking systems.

ESBs analyze and store samples in order to understand the effects of chemicals, emerging substances and the environmental change in biota. For a correct analysis of the effect induced by these variables, there is a need to add biological endpoints, such as biomarkers, to the endpoints based on chemical approaches which have been used alone in the past. Thus, research efforts must be addressed to analyze the biological endpoints for the assessment of the biological effects in the frame of ESB. This would result in thorough investigations of the applied ESB routine at three levels, i.e., sampling, sample processing and analyzing, and archiving (Garmendia et al., 2015). It is suggested that ESBs consider adapting their sampling strategies so that the stored samples can comply with novel tools which are developed for the integrated exposure and effects analysis. In that way, in the future, this would enable scientists to apply new biological methods in a consistent way to ESB samples and generate time trends that integrate exposure and effects parameters.

The combination of chemical and biological endpoints has been already successfully applied in marine pollution monitoring programs to determine the long term biological effects of pollutants (Garmendia et al., 2011). In fact, over the next decade, environmental samples management will be likely to focus equally on biological and ecological conditions rather than chemical conditions, with ecosystem health at center of regulation management decision making (Apitz et al., 2006). Consequently, banking practices and strategies need to be adapted to archive specimens suitable to measure biological effect in order to assess the environmental health status.

It is clear that the necessity of ESBs to add biological endpoints is fundamental (e.g., assessment of the environmental health status). Moreover, advances and development of high sensitive, high-throughput techniques along with ecotoxicological approaches based on biomarkers are stimulating a new demand for stored specimens and associated data. That is why, like in chemically targeted

environmental specimen banking, the banked samples for the assessment of biological effects also will require guidance informed by knowledge of their practices and challenges, along with policies for the correct advancement of research goals and appropriate and effective biobank governance. In this way, one of the objectives of the present work is to provide an overview on the sampling strategy and sample processing conventionally used for chemical endpoints by different ESBs in order to obtain biological endpoints through biomarkers (**Table 2**).

Table 2. Processing and Storage methods of the most relevant ESBs worldwide.

Environmental Specimen Bank (ESB)	Processing and Storage methods	Analysis
NIST Marine ESB *	Specimens selected for analysis are cryogenically homogenized in an adjoining ISO Class 5 clean room and divided into aliquots for analysis and/or continued cryogenic storage.	Chemical analyses.
German ESB	Crushing as processing method. Samples are stored in liquid nitrogen vapor (-150 °C) freezers with continuously monitored security systems.	Chemical analysis
Swedish ESB	Most samples stored frozen at –30º C and –80º C. Some samples stored dry (lyophilized) at room temperature.	Chemical analysis
Canada National Wildlife Specimen Bank	Samples are preserved in an environmentally controlled facility at −40°C and −80°C in a series of freezers and liquid nitrogen vapour phase.	Chemical analysis
Canada´s National Aquatic Biological Specimen Bank	Cryo-preserved in an environmentally controlled facility at −80°C in a series of freezers.	Chemical analysis
Japan es-Bank	Cryogenic storage with four ultra cold –80°C electric freezers and three liquid nitrogen vapor freezers with continuously monitored security systems	Molecular analysis
Japan Time Capsule Program **	Ball-milling as processing method. Long-term storage using liquid nitrogen vapor (-150°C)	Chemical analysis

(*)**Figure 1**   (**)**Figure 2**

Figure 1. Cryogenic storage at NIST Marine Environmental Specimen Bank (http://www.nist.gov/mml/csd/esb/marineesb.cfm)

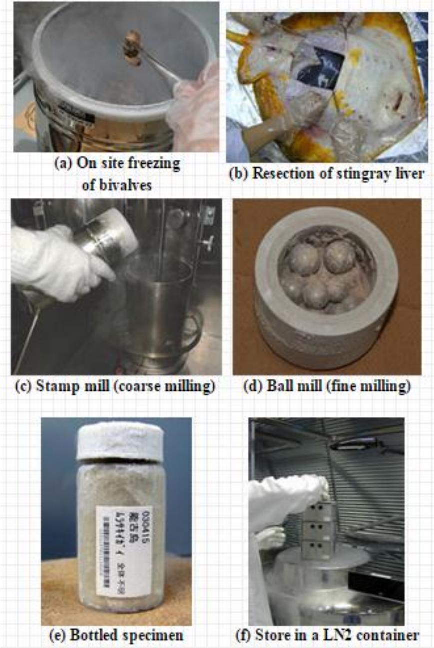

Figure 2. Processing method of samples, Time Capsule for Environmental and Endangered Wildlife (Japan) (http://www.nies.go.jp/timecaps1/summary/objectiveE.htm)

One of the most used biological endpoints for the assessment of the environmental health status are biomarkers which can be defined as measurable changes in biological responses at molecular, biochemical, cellular, physiological and behavior levels. Biomarkers responsiveness can be related to exposure to pollutants (exposure biomarkers) or to toxic effects of environmental chemicals (effect biomarkers) (McCarty and Shugart, 1990; Peakall, 1992; Van der Oost et al., 2003). The application of biomarkers is particularly relevant since molecular and/or biochemical alterations may indicate the occurrence of environmental disturbance at an early stage, before a more integrates form of toxicity appears at higher levels of the biological organization. So, changes at simple levels of biological organization (e.g., molecule, cell, and tissue) can anticipate changes at more complex levels (e.g., population, community, and ecosystem) (Moore et al., 2004; Galloway et al., 2006). Thus, biomarkers are increasingly discussed as short-term indicators of long-term biological effects (McCarthy and Shugart 1990; Cajaraville et al., 2000; Garrigues et al., 2003).

At biochemical level two of the most currently used biomarkers are the activity of acetylcholinesterase (AChE) and glutathione S-transferases (GST) enzymes. Acetylcholinesterase (AChE) is an essential enzyme in the transmission of the nerve impulse that degrades acetylcholine to choline and acetic acid in the synaptic gap of

cholinergic synapses and neuromuscular junctions (Vidal-Liñan et al., 2013). AChE has important functions in cholinergic neurotransmission, sequestration and hydrolysis of several xenobiotics and natural substances. They are strongly inhibited by organophosphate and carbamate insecticides and they have been widely used as specific biomarkers for these compounds. Recently, a more broad use of these enzymes as environmental biomarkers has been done since several studies showed that they are also inhibited by other environmental contaminants, such as some metals, synthetic detergents, undetermined components of fuel oils and complex mixtures of pollutants at concentrations that may be ecologically relevant (Labrot et al., 1996; Payne et al., 1996; Guilhermino et al., 1998; Moreira et al., 2004; Moreira and Guilhermino, 2005; Vieira et al., 2008). Thus, the inhibition of AChE activity in mussels has been frequently used as a biomarker of chemical pollution by metals and pesticides (Bocquené and Galgani, 1990).

GST is an important family of phase II biotransformation enzyme involved in the conjugation and detoxification of organic compounds, which also play a protective role against oxidative stress by catalyzing a selenium independent glutathione peroxidase activity (Prohaska, 1980; reviewed by Sheehan et al., 2001). Since the induction of the activity of these enzymes by several groups of pollutants, including PAHs and petrochemical products, is well established, they have been widely used as environmental biomarkers in estuaries and coastal areas (Martínez-Gómez et al., 2006; Lima et al., 2007; Guimarães et al., 2009).

Mussels were used as sentinel species because they are indicators of environmental health status. They constitute commercially important sea-food species on a worldwide basis and thus there is an economic and public health concern about them. But they are also key organisms in marine pollution monitoring programs and biobanking activities as well as in laboratory experiments.

Mytilus galloprovincialis (Lamarck, 1819) known as black mussel, belongs to Phylum Mollusca, Class Bivalia and Order Mytiloida (MSIP, 2009) (**Figure 3**).

Figure 3. ***Mytilus galloprovincialis.***

In ecotoxicology mussels are model sentinel species since they are filter-feeders with very low metabolic activity, which implies that the pollutants concentrations in their

tissues reflect more accurately the environmental pollution magnitude (Widdows and Donkin, 1992). Moreover, they are relatively tolerant (but not insensitive) to a wide range of environmental conditions, including moderately high levels of most types of contaminants. They are economically relevant sessile organisms that are widely distributed, being easy their sampling. Mussels are also easy to maintain in acceptable conditions in laboratory, making possible their very extensive use in experimentation (Bayne, 1989; Phillips and Rainbow, 1989).

3. HYPOTHESIS

The hypothesis of the present work is that biochemical biomarkers can be measured in samples archived in ESBs that have been routinely processed for chemical analysis. In that way, the ESBs worldwide could afford a new methodological approach to obtain biological endpoints (biomarkers of exposure and effect) in samples stored for decades, and could represent an appropriate tool for retrospective analysis that help the ecosystem health assessment.

4. OBJECTIVES

The main objective of the present work is to measure AChE and GST enzymatic activities in different tissues of mussels, *Mytilus galloprovincialis*, including foot, digestive gland, gill, gonad and whole soft tissue (both pooled and beaten and lyophilized). It is important to note that the samples stored in the BBEBB and some of the ESBs worldwide are previously pooled and beaten or lyophilized, as a standard protocol of chemical analysis, and therefore, a special effort to adapt the protocols developed in the fresh samples has been done. This main objective is divided in four specific objectives:

- To prove the suitability of samples stored in ESBs for chemical analysis to apply biological endpoints through biochemical biomarkers.
- To measure the enzymatic activity in pooled and beaten and lyophilized samples.
- To compare AChE and GST activity and protein levels between each of the tissues dissected, pooled and lyophilized samples.
- To compare the enzymatic activity and protein levels between the mussels from the two localities (Plentzia and Arriluze) with different pollution history.

5. MATERIAL AND METHODS

5.1 EXPERIMENTAL DESIGN

In April 2014 and March 2015 30 mussels were handpicked from two localities along the NE coast of Biscay. These sites were selected since differences in biochemical responses in dissected tissues of mussels (*Mytilus galloprovincialis)* have been previously observed. Plentzia (43°24'49''N, 28°56'45''W) is a locality typically used as a clean point. And the leisure harbour of Arriluze (43° 82´49´´N, 28°56´9´´W), in the Abra estuary in the Basque Coast, is a site known by its high levels of pollution (Orbea et al., 2006; Ortiz-Zarragoitia et al., 2012; Lekube et al., 2014).

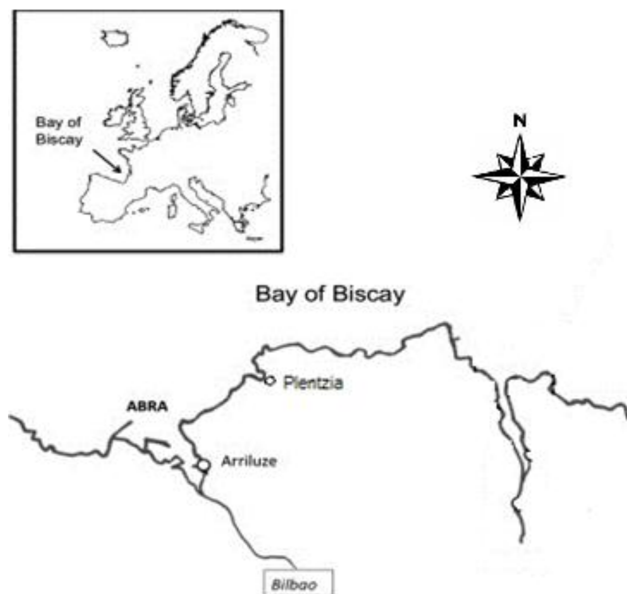

Figure 3. Map of sampled sites. Mussels were collected at two localities from the Bay of Biscay. The leisure harbor Arriluze, in the Abra estuary, was sampled as polluted external site. Plentzia was sampled as a reference site.

From each sampling site, the tissues gill, foot, gonad and digestive gland of 5 mussels were dissected out, isolated in cryovials, immediately frozen in liquid nitrogen (-196°C) and stored at -80°C for later biochemical analyses. The soft tissue of 10 mussels were removed, pooled, beaten with a blender and stored in liquid nitrogen (-196°C) into 2 groups of each locality for biomarker analysis. The remaining 10 mussel tissues lyophilized at 2mB and -50°C during 14h using a Varian DS102 vacuum pump. The lyophilized samples were stored at 4°C in 2 falcons of each locality until required for analysis.

These methodologies have been chosen since they are the two processing methods used in the BBEBB (Biscay Bay Environmental Biospecimen Bank) for chemical analysis.

5.2 ENZYMATIC ASSAYS

Gill, foot, gonad, digestive gland, pooled (in frozen conditions one sample of each pool was taken with tweezers) and lyophilized samples were defrost, weighed and homogenized (PRECELLYS®24/PRECELLYS®24-DUAL lyser/homogenizer) at a 1:4 w/v ratio using 100 mM phosphate buffer (pH 7.4) at 6000 rpm for 30 seconds at 6°C and centrifuged at 12000 rpm for 20 min at 4°C, using a Beckman Coulter Microfuge 22R centrifuge (along all the process the samples were maintained on ice between the different steps to ensure their perfect conservation and avoid the action of the proteases).

After the homogenization process the supernatants of all the samples were collected and divided into aliquots. The aliquots stored at -80°C until the measurements of protein concentration and enzymatic activities.

5.2.1 Determination of the protein concentration of the samples

The protein concentration of the samples was determined by the Bradford method (Bradford, 1976) adapted to microplate, using bovin serum albumin (BSA).

The Bradford assay is a protein determination method that involves the binding of Coomassie Brilliant Blue G-250 dye to proteins (Bradford, 1976).The dye exists in three forms: cationic (red), neutral (green), and anionic (blue) (Compton and Jones, 1985). Under acidic conditions, the dye is predominantly in the doubly protonated red cationic form (Amax = 470 nm). However, when the dye binds to protein, it is converted to a stable unprotonated blue form (Amax = 595 nm) (Fazekes de St. Groth et al., 1963; Reisner et al., 1975; Sedmack and Grossberg, 1977). It is this blue protein-dye form that is detected at 595 nm in the assay using a microplate reader. Work with synthetic polyamine acids indicates that Coomassie Brilliant Blue G-250 dye binds primarily to basic (especially arginine) and aromatic amino acid residues (Compton and Jones, 1985). Spector (1978) found that the extinction coefficient of a dye-albumin complex solution was constant over a 10-fold concentration range. Thus, Beer's law may be applied for accurate quantitation of protein by selecting an appropriate ratio of dye volume to sample concentration.

In any protein assay, the ideal protein to use as a standard is a purified preparation of the protein being assayed. In the absence of such an absolute reference protein, another protein must be selected as a relative standard. The two most common protein standards used for protein assays are BSA and gamma-globulin. With the Quick Start Bradford protein assay, dye color development is significantly greater with BSA than with most other proteins, including gamma-globulin (Quick Start ™ Bradford Protein Assay, Instruction manual, BIO-RAD).
The concentration of BSA standards used were 0.125, 0.5, 0.75, 1, 1.5 and 2 mg/ml, so in order to be able to know the protein concentration of each sample the supernatants were normalized to approximate protein concentration of 0.7 mg/ml, using 100 mM phosphate buffer (pH 7.4). After many trials these dilutions were determined as follows: gill was diluted at 1:10 w/v ratio, foot was diluted at 1:10 w/v ratio, gonad was diluted at 1:10 w/v ratio and digestive gland was diluted at 1:20 w/v ratio. The pooled and beaten and the lyophilized samples were diluted at 1:200 w/v ratio.

All determinations were performed in a microplate reader Bio Tek Eon™ High Performance Microplate Spectrophometer, at 25°C.

5.2.2 Glutathione S-Transferase activity (GST)

Glutathione S-transferase (GST) was selected as biomarker of antioxidant defence and/or detoxification. The activity of GST was determined in gill, gonad, foot, digestive gland, pooled and lyophilized samples according to Habig et al (1974) adapted to microplate by Frasco and Guilhermino (2002).

The final concentration for the GST assay, in a final volume of 300 µL, were 10 mL potassium phosphate buffer (0.1 M; pH 7.4), 300 µL GSH and 300 µL 1 chloro-2,4-dinitrobenzene (CDNB). The activity of GST was determined by measuring the formation of a thioether by the conjugation of CDNB with GST. This conjugation is followed by an increase in absorbance at 340 nm, using the microplate reader Bio Tek Eon™ High Performance Microplate Spectrophometer.

5.2.3 Acetylcholinesterase activity (AChE)

Acetylcholinesterase (AChE) activity was quantified in gill, foot, gonad, digestive gland, pooled and lyophilized samples to assess mussels' neurotransmission levels.

The activity of AChE was determined according to Ellman et al (1961), adapted to microplate by Guilhermino et al (1996).

The final concentration of the assay, in a final volume of 300 µL, was: 200 µL acetylthiocholine ioide (ATCh) 0.075 M, 1 mL DTNB 10 mM and 30 mL phosphate buffer (0.1 M; pH 7.2). In this assay the AChE hydrolyses the substrate ATCh in thiocholine and acetate. Following this reaction, the thiocholine reacts with DTND forming a mixed disulphide and the yellow chromophore 5-thio-2nitobenzoic acid (TNB). The TNB formation is followed by an increase in absorbance at 412 nm, using a microplate reader. Eon^{TM} High Performance Microplate Spectrophometer.
All enzymatic assays were performed at 25°C. AChE activity is inhibited at 19°C, but this inhibition is not significant at 24 °C (Vidal-Liñan and Bellas, 2013).

5.3 STATISTICAL ANALYSIS

SPSS v 17.0 software (SPSS INC., Chicago, Illinois) was employed for the statistical analyses. Normality of data (Kolmogorov-Smirnov's) and homogeneity of variance (Levene's test) were tested before statistical analyses. Statistically significant differences among the dissected tissues were tested according to the Duncan's post-hoc test based on one-way analysis of variances (one-way ANOVA) for parametric variables (Protein concentration, GST and AChE). The differences between localities were tested according to the Student t-test for parametric variables (Protein concentration, GST and AChE). The differences between the three processing methods were tested according to the Z-score for parametric variables (Protein concentration, GST and AChE). A 95% significance level ($p < 0.05$) was established for all statistical analyses carried out.

6. RESULTS

The samples obtained by the three different methods of sampling processing that Environmental Specimen Banks usually use (dissected tissues, pooled and lyophilized) were homogenized following the same protocol. When dissected tissues were processed no problem was observed, except that the muscle was impossible to homogenize due to its high elasticity. No problem was observed in the homogenization of the pooled samples. The homogenization of the lyophilized samples was repeated several times in order to obtain a good result. Protein concentration, activity of acetylcholinesterase and activity of GST were measured in each sample.

6.1 PROTEIN CONCENTRATION

Values of protein concentration in the four different dissected tissues (digestive gland, gill, foot and gonad) and in the pooled and lyophilized samples are presented in **Figure 4.** The protein concentration in the pooled samples is higher than the protein concentration in the dissected tissues. The protein concentration in the lyophilized samples is the highest one in both localities (**Figure 4A, 4B**).

After the comparison between the four different dissected tissues (**Figure 4C**), in mussels from Plentzia the digestive gland (DG) is statistically the tissue with the highest protein concentration, followed by gonad (GD), gill (G) and foot (F). In mussels from Arriluze the digestive gland (DG) is the dissected tissues with statistically more AChE activity, followed by gill (G), gonad (GD) and foot (F).

Significant differences were found in the protein concentration of all the dissected tissues between mussels from Plentzia and Arriluze (**Figure 4C**).

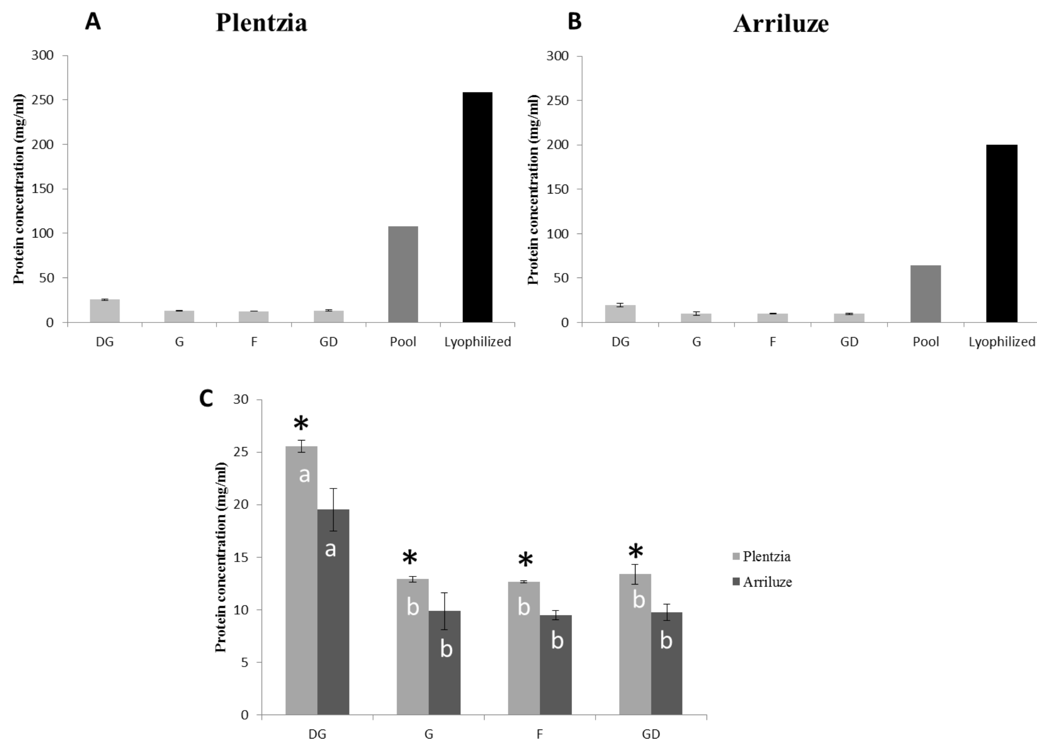

Figure 4. (A) (B) Protein concentration in *Mytilus galloprovincialis* digestive gland (DG), gill (G), foot (F), gonad (GD), pooled samples (Pool) and lyophilized samples (Lyophilized) from Plentzia and Arriluze. (C)Detailed of protein concentration of *Mytilus galloprovincialis* digestive gland (DG), gill (G), foot (F) and gonad (GD). Bars represent means of protein level (n=5 in the tissues; n=10 in the pooled and lyophilized samples) and the intervals correspond to standard errors. Asterisks indicate significant differences among the two localities according to the T-test (p <0.05). Letters indicate significant differences between the four dissected tissues according to the Duncan test after a one-way Anova (p <0.05).

6.2 GLUTATHIONE S-TRANSFERASE ACTIVITY (GST)

Values of GST activity in the four different dissected tissues (digestive gland, gill, foot and gonad) and in the pooled and lyophilized samples are presented in **Figure 5.** The GST activity is higher in the dissected tissues than in the pooled and lyophilized samples, which are formed by 10 individuals. The enzymatic activities of lyophilized and pooled samples are below 0.5 nmol/min*mg prot. The gonad presents the highest GST activity levels compared to other tissues and the other different processing methods.

After the comparison between the four different dissected tissues (**Figure 5C**), the gonad (GD) is statistically the tissue with more GST activity, followed by gill (G), foot (F) and digestive gland (DG).

Significant differences were found in the GST activity between the foot (F) of mussels from Plentzia and the foot (F) of mussels from Arriluze (**Figure 5C**).

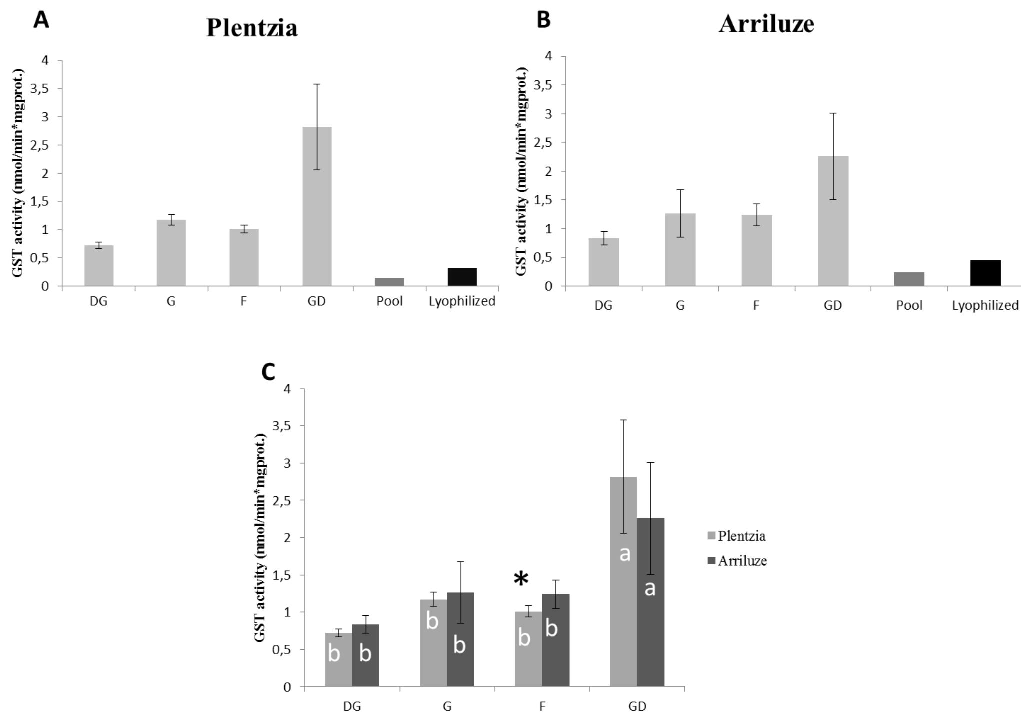

Figure 5. (A) (B) Glutathione S-transferase activity in digestive gland (DG), gill (G), foot (F), gonad (GD), pooled samples (Pool) and lyophilized samples (Lyophilized), respectively, collected from the two sampling stations Plentzia and Arriluze. (C) Detailed of GST activity of *Mytilus galloprovincialis* digestive gland (DG), gill (G), foot (F) and gonad (GD). Bars represent means of protein level (n=5 in the tissues; n=10 in the pooled and lyophilized samples) and the intervals correspond to standard errors. Asterisks significant indicate differences among the two localities according to the T-test (p <0.05). Letters indicate significant differences between the four dissected tissues according to the Duncan test after a one-way Anova (p <0.05).

6.3 ACETYLCHOLINESTERASE ACTIVITY (AChE)

Values of AChE activity in the four different dissected tissues (digestive gland, gill, foot and gonad) and in the pooled and lyophilized samples are presented in **Figure 6.** The AChE activity is higher in the dissected tissues than in the pooled and lyophilized samples, which are formed by 10 individuals. The enzymatic activities of lyophilized and pooled samples are below 0.5 nmol/min*mg prot. The gill of mussels from Plentzia and the gonad of mussels from Arriluze present the highest AChE activity levels compared to other tissues and the other different processing methods.

After the comparison between the four different dissected tissues (**Figure 6C**), in mussels from Plentzia the gonad (GD) is the tissue with more AChE activity, followed by foot (F), gill (G), and digestive gland (DG). In mussels from Arriluze the gill (G) is the dissected tissues with more AChE activity, followed by gonad (GD), foot (F) and digestive gland (DG). The digestive gland (DG) is statistically the tissue with less AChE activity.

Significant differences were found in the AChE activity between the digestive gland (DG) of mussels from Plentzia and the digestive gland (DG) of mussels from Arriluze (**Figure 6C**).

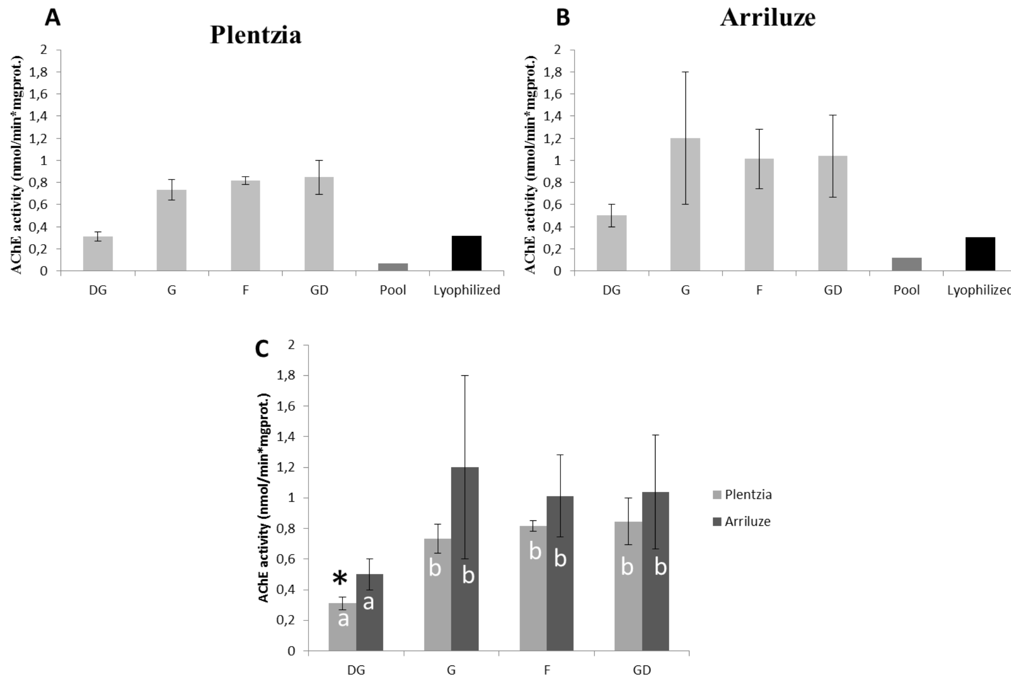

Figure 6. (A) (B) AChE activity in digestive gland (DG), gill (G), foot (F), gonad (GD), pooled samples (Pool) and lyophilized samples (Lyophilized), respectively, collected from the two sampling stations Plentzia and Arriluze. (C)Detailed of GST activity of *Mytilus galloprovincialis* digestive gland (DG), gill (G), foot (F) and gonad (GD). Bars represent means of protein level (n=5 in the tissues; n=10 in the pooled and lyophilized samples) and the intervals correspond to standard errors. Asterisks indicate significant differences among the two localities according to the T-test (p <0.05). Letters indicate significant differences between the four dissected tissues according to the Duncan test after a one-way Anova (p <0.05).

6.4 PLENTZIA vs. ARRILUZE

For the comparison of the three different sampling processing methods of mussels from the two localities, among the four tissues, digestive gland was chosen as significant differences were found for this tissue (**Figure 7**).

The protein concentration in mussels from Plentzia is significantly higher than in mussels from Arriluze, in all the processing methods (**Figure 7A**).

Differences in GST activity between mussels from Plentzia and mussels from Arriluze were obtained. Mussels collected in Arriluze, present the highest values of GST activity (**Figure 7B**). Except for the gonad tissue, which follows the contrary trend, as the GST activity in the gonad tissue is slightly higher in mussels from Plentzia (**Figure 5C**).

The AChE activity levels in mussels from Arriluze are higher than in mussels from Plentzia, except in the lyophilized samples, where the mussels from Plentzia present a higher AChE activity than the mussels from Arriluze (a difference of 0,011 nmol/min*mg prot.) (**Figure 7C**). Protein concentration and GST activity follows the same pattern for the three different processing methods. In the AChE activity only two processing methods (dissected tissues and pooled) follow the same pattern.

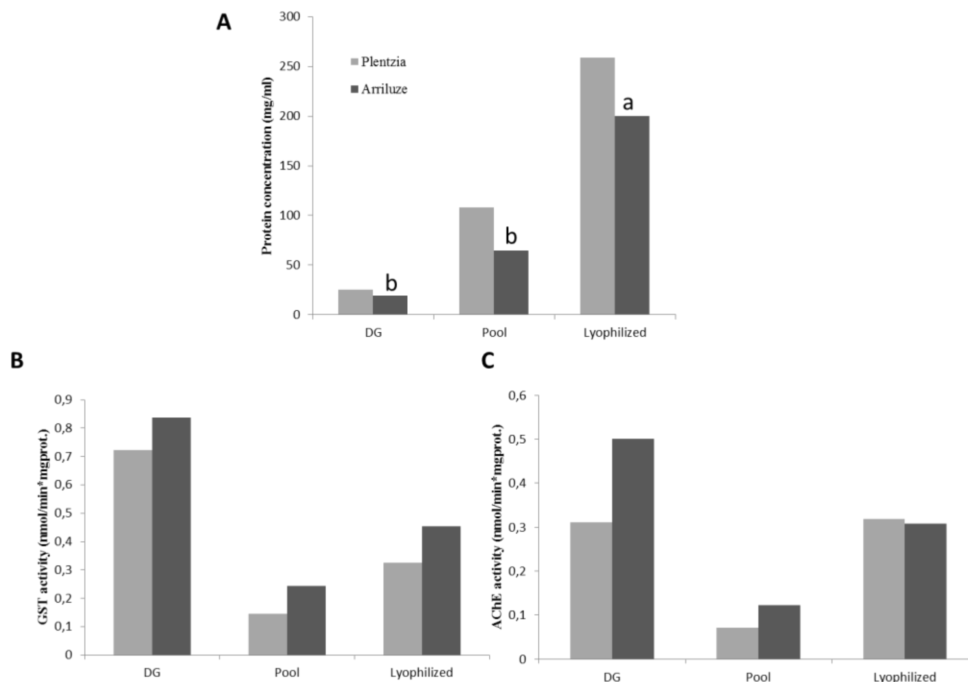

Figure 7. Comparison between the mussels from the two localities Plentzia and Arriluze. (A) Comparison of the protein concentration in digestive gland, pooled and lyophilized samples. (B) Comparison of GST activity in digestive gland, pooled and lyophilized samples. (C) Comparison of AChE activity in digestive gland, pooled and lyophilized samples. Bars represent means of protein level (n=5 in the tissues; n=10 in the pooled and lyophilized samples) and the intervals correspond to standard errors. Letters indicate significant differences among the three processing methods according to the Z-score (p <0.05).

7. DISCUSSION

In the present study, enzymatic activities were measured in different tissues of mussels, *Mytilus galloprovincialis*, including foot, digestive gland, gill, gonad and whole soft tissue (both pooled and beaten and lyophilized). The aim was to assess the possibility to measure protein concentration and enzymatic activity in the three processing methods. The different levels of protein concentration and the enzymatic activity in each processing methods and between the two localities was also compared. Most of the ESBs worldwide usually process their samples in order to carry out solely chemical analysis. With that objective some ESBs pooled and beaten or lyophilized their samples before being stored, as the BBEBB (Biscay Bay Environmental Biospecimen Bank) normally did. In the present work biochemical biomarkers were applied to these kinds of samples. In that way, a new opportunity could be open to carry out retrospective studies regarding biological endpoints in those ESBs that are, up to now, mainly focused on chemical analysis.

As far as we know, the present study is the first one to report the measurements of protein concentration and application of biomarkers (AChE and GST) in samples processed for chemical analysis in ESBs worldwide. Moreover, the present study proved that biochemical analysis can be done in pooled and beaten and lyophilized samples as will be thoroughly discussed below.

In the homogenization step (Material and Methods, section 4.2) the sample which rendered the best homogenization (no solid matters appeared after the homogenization) was the foot. The lyophilized samples were the most difficult ones to homogenize probably due to the fact that the water was removed from the sample during the lyophilization process. Thus, in this case, the homogenization starts with less water than the other samples. The muscle was impossible to homogenize, because the muscle is a very rigid tissue and very difficult to rip. During the centrifugation and after doing the biochemical analyses of protein concentration and enzymatic activity no methodological differences were appreciated between all the samples.

Results obtained in the present study show that the protein concentration in the pooled and in the lyophilized samples is higher than the protein concentration of any of the individual tissues. As in the pooled samples and lyophilized samples the whole tissue is taken, proteins that do not present enzymatic activity like collagen also are measured. Consequently, these samples present the lowest enzymatic activity since the results of the enzymatic levels (similar in all the samples) are standardized with the protein concentration.

Sensitivity is one of the main requirements for a biomarker to be used in monitoring programs (Sanchez and Porcher, 2009). In the selection of the target organ of *Mytilus galloprovincialis* for the development of a useful biomarker, besides taken into account the grade of facility to process each one, the sensitivity and the magnitude of response of the organ is also an important factor to consider. Results of the present study indicate that the lowest GST activity was measured both in the pooled and lyophilized samples. In the comparison between the dissected tissues the highest GST activity was found in the gonad followed by the gill, foot and digestive gland of mussels. In the same way, Regoli and Principato (1995) reported that the GST activity in the gill of *M. galloprovincialis* was higher than in the digestive gland. Vidal-Liñan et al (2013) indicated also that GST and AChE activities were higher in the gill than in the digestive gland of mussels.

The AChE activity is also quite low in the pooled and lyophilized samples compared with the enzymatic activity levels of the dissected tissues. The AChE activity in mussels from Plentzia was the highest also in the gonad, followed by the foot, gill and digestive gland. Tough, it was found that in mussels from Arriluze the highest AChE activity was on gill, followed by gonad, foot and digestive gland. In agreement with the present results, Mora et al (1999) reported that the AChE activity in the gill of *M. galloprovincialis* was about four times higher than in the other organs, whilst the lowest activity was found in the digestive gland. Brown et al (2004) demonstrated that the highest specific activity of AChE was found in foot of the common mussel, *Mytilus edulis* and gill showed the second highest specific activity. In the present work, digestive gland was chosen as the most suitable tissue fraction for the comparison between the different processing methods as significant differences were found in this tissue.

It has to be mentioned that in previous studies mentioned above (Regoli and Principato, 1995; Vidal-Liñan et al., 2013; Mora et al., 1999 an Brown et al., 2004) the AChE and GST activities were not measured in gonad. In the present work the greatest activity of these biomarkers was recorded in this tissue maybe related to the high variability associated to the gonadal cycle (the mussels from which the gonads were dissected were collected in April). In the present study, enzymatic activity was measured in all the possible target organs since the main aim was to prove that it was feasible to know the values of AChE and GST activities in samples stored by different processing methods.

Except for the lyophilized samples that presented a slightly higher AChE activity in mussels from Plentzia than in mussels from Arriluze, mussels from Plentzia exhibited the lowest values of enzymatic activity of GST and AChE. The protein concentration in mussels from Plentzia was higher than in mussels from Arriluze, in all the processing methods, as mussels from Arriluze (a polluted area) present a

higher concentration of neutral lipids than mussels from Plentzia (Marigomez et al., 2013). However, the differences between the levels of each variable (protein concentration, AChE and GST activity) in the two localities are not so significant. This could be related again to the season in which mussels were collected. Thus, mussels from different seasons should be collected, in order to investigate how the levels of the variables change during the year. Protein concentration and GST activity follow the same pattern between mussels from Plentzia and mussels from Arriluze processed by the three different processing methods. This is in agreement with the expected degree of contamination between both localities. The increase in GST activity in mussels from Arriluze means that Arriluze is a more polluted area than Plentzia. Accordingly, highest GST values are found in mussels collected in stations potentially contaminated (Moreira and Guilhermino, 2005). In this way, regardless of the processing method used for the sample, the levels of GST activity and protein concentration can differentiate a contaminated site from a clean one. These results are in concordance with the assumption made in the beginning that Plentzia is a good reference site. In this way, these parameters could be used for retrospective studies of biomonitoring.

Nevertheless, intricate results have been observed in the levels of the enzymatic activity of AChE. This controversy has been observed in previous studies like in Chen et al (2012) who reported that this enzyme activity was significantly inhibited in larvae derived from the adult zebrafish (*Danio rerio*) exposed to 0.16, 0.8 and 4 µg L-1 of PBDE, compared with controls. In other study with the Artic spider crab (Minier et al., 2008), muscle AChE activity was significantly inhibited in individuals exposed to DPA, BPA and BPDE during 3 weeks. On the contrary, Key et al (2009) observed that AChE activity from the estuarine fish (*Fundulus heteroclitus*) showed values significantly higher than controls in the lowest concentration of 0.0125 mg L-1 of PBDE.

In relation to the storage methods the present study has proven that in the way ESBs store their biological samples, the AChE and GST biomarkers could be measured on samples archived for a long time as pooled or lyophilized samples. Therefore, they can be processed in order to make biochemical analysis. However, when analyzing biochemically, some problems have been found. Even if the ESBs continue to process their samples by pooling and beating and lyophilizing them, instead of storing them in high capacity containers it could be suggested to split samples in smaller aliquots to make easier the biochemical assays. The problem is that the processing of a big pool sample, needs a prior defrost to obtain just a small subsample. In the defrosting process the enzymes in the sample can denaturize or lose some of their activity, so in the future the results of biochemical measurements would not be so accurate. When doing chemical analyses the defrosting is not a major problem. In order to be able to do biochemical assays, it would be preferable

to store the pool in small eppendorfs with the necessary amount to measure the protein content and the enzymatic activity of the pools. In the case of the lyophilized samples, it does not matter how the ESBs store them because these samples are maintained at 4ºC, meaning that there is no risk of defrosting the whole sample. Samples could be stored individually and in frozen conditions instead of pooling them and, in that way, be processed with the most appropriate method for each analysis, make statistics or observe the differences between males and females, for instance. Another factor that has to be taken into account when analysing these processing methods is that in the lyophilized samples and the pooled samples the whole soft tissue is selected. Therefore, some proteins of the muscle and other proteins of the *Mytilus* like collagen, which did not appeared in the dissected tissues, could be measured. Lyophilized samples showed a highest protein concentration because they are dried but they were diluted following the same protocols as the other wet samples. Because of this the results have not been so accurate. However, it has been possible to measure protein concentration and biochemical biomarkers (AChE and GST) in pooled and lyophilized samples. This problem could be solved by characterizing these types of samples.

In the present work, as mentioned before, it is possible to compare the enzymatic activity between the different tissues and the lyophilized and pooled samples. However, a problem not associated with the procedure was detected, that is, the gonadal cycle of the *Mytilus galloprovincialis* has to be taken into account. For instance, the mussels from which the different tissues were dissected were collected in April, whereas, the individuals to make a pool and to lyophilize them were collected in February. In April the mussels are spawning, whereas in February the gonads´ follicles are full of mature gametes (Suárez et al., 2005). Radenac et al (1998) pointed out to the possibility that the assessment of AChE activity is disturbed by the spawning process, because the release of gametes might also lead to a loss of protein, without an equivalent decrease in the concentration of AChE. Moreira and Guilhermino (2005) reported that a seasonal variation in AChE and GST activities was found.

Nowadays, standardized protocols exist to measure the enzymatic activity of AChE and GST in the different tissues of the mussel *Mytilus galloprovincialis* (ICES 2012). However, there are no for the measurement of enzymatic activity in lyophilized and pooled samples. Presently, significant differences appeared between the two localities in the dissected tissues, but no significant differences were found between the two localities in pooled and lyophilized samples. The next step should be to characterize the biomarkers in these samples, in order to determine which values of the enzymatic activity correspond to contaminated samples. With that objective a retrospective could be done with samples from different ESB to observe how the

GST and AChE activity levels change over a year, and compare that data with the results obtained in the biochemical analysis of dissected tissues.

In the future, if biological endpoints as well as chemical analysis are going to be measured in the samples, the sampling strategy and sampling processing used by different ESBs should be modified according to the endpoints to be measured. Even if it is possible to analyze biochemically pooled and lyophilized samples, as the present study has proven, some problems in the procedure have to be outlined: a) ESBs store their pooled and lyophilized samples in high capacity containers and therefore, a subsequent defrost to obtain the subsamples is needed. The solution could be to store the pool in small eppendorfs with the necessary amount to measure the protein content and the enzymatic activity of the pools; b) the individuals are pooled so it is not possible to make statistics or see the differences between males and females. To solve this problem samples could be stored individually and in frozen conditions; c) in the pooled and lyophilized samples the protein concentration is extremely high because the whole soft tissue is taken and in the lyophilized samples the water is removed from the sample and the homogenization starts with less water than the other samples. The solution to this problem could be to characterize these samples or instead of standardized the results of their enzymatic levels with the protein concentration, standardized with the shell´s weigh or the condition index.

In conclusion, the results obtained in the present study are promising for the ESBs worldwide to apply new biological methods to the samples stored for decades. The approach used in this work can be improved in the future by taken samples for each season of the year, in order to characterize the samples processed for chemical analysis in the ESBs. This way, not only chemical endpoints but biochemical endpoints could also be used to assess the health of the environment.

8. CONCLUSIONS

- Protein concentration and enzymatic activity of two biochemical biomarkers (AChE and GST) can be measured in samples processed as pooled and lyophilized for chemical analysis.

- Digestive gland is the most suitable tissue fraction for the comparison between the different processing methods.

- AChE and GST activity and protein levels vary between the tissues dissected and pooled and lyophilized samples.

- Some problems which could be solved have been detected in the suitability of samples stored in ESBs for chemical analysis when applying biological endpoints.

- Even in the pooled and beaten and lyophilized samples trends are detected between Plentzia and Arriluze when measuring protein concentration and GST activity.

- The pooled and beaten and lyophilized samples should be characterized in order to be able to determine which values of the enzymatic activity correspond to contaminated samples.

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