Hepatic biomarkers of xenobiotic metabolism in eighteen marine fish from NW Mediterranean shelf and slope waters in relation to some of their biological and ecological variables

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Abstract

A suite of hepatic biomarkers currently used in pollution monitoring were measured in eighteen common fish species, comprising five orders, eleven families of teleosts and two elasmobranchs. The sampling was carried out seasonally in front of the Barcelona coast (NW Mediterranean) during 2007. The hepatic enzymes considered were the activities of catalase, glutathione reductase, ethoxyresorufin *O*-deethylase, carboxylesterase and glutathione S-transferase. As markers at higher levels of biological organization, feeding preferences (on benthic, suprabenthic or zooplanktonic species), swimming capability, stomach fullness and trophic level were considered. Significant species differences were found among all the biochemical parameters analysed, although no relationships among the biomarkers themselves were evidenced. In general enzymatic activities were much higher in teleosts than in elasmobranchs, and in perciforms than in gadiforms. Seasonality was observed in some species with higher activities usually corresponding to the winter period. No site related differences were observed in the two selected sites, which differ over a small pollution gradient. A multivariate canonical correspondence analysis (CCA) was performed on shelf and slope species separately to relate biochemical markers with ecological variables. CCA revealed that for shelf species, EROD was positively related to benthos feeding as well as trophic level, while on the slope the clearest association was between suprabenthos feeders and trophic level. Our present results, including seasonality, slightly differ from former observations (Solé et al., 2009a) and reveal a more significant role of the ecological variables in controlling biomarkers expression in fish from the shelf.

Keywords: antioxidant enzymes, xenobiotic metabolism, ecological parameters, marine fish, NW Mediterranean

1. Introduction

In the marine environment, pollution monitoring studies are currently carried out mainly in coastal areas due to their proximity to the point sources of anthropogenic chemicals. However, transport of chemicals from coastal sites to greater depths by sea currents as well as the global atmospheric transport, contribute to the presence of pollutants offshore and in remote areas (UNEP/MAP, 2001). The NW Mediterranean shelf and slope are subject to intense fisheries, and the occurrence of anthropogenic chemicals in their waters/sediment/biota has been well studied within the Mediterranean context (Albaigés et al., 2005; Gómez-Gutiérrez, 2007). Nevertheless, studies reporting the effects of chemicals on commercial fish are scarce as most of them focus on the sentinel red mullet in coastal sites (Porte et al., 2002; Martin-Skilton et al., 2006; Zorita et al., 2008), with less attention being paid to species from fishing grounds (García de la Parra et al., 2000; Solé et al., 2006; 2009a; 2009b). Thus, we have investigated the presence of organic pollutants (PAHs, PCBs and DDTs) in sediment from two areas: shelf and slope. We have also determined the activities of hepatic markers involved in the metabolism of foreign compounds (xenobiotics), as well as antioxidant defences, in several fish species from these two habitats. The biomarkers selected are among those currently adopted in pollution monitoring studies (Cajaraville et al, 2000; van der Oost et al., 2003). The main role of the antioxidant defences is to prevent the action of an excess of oxyradicals resulting from exposure to xenobiotics (Valavanidis et al., 2006). Among the antioxidant defences, catalase (CAT) transforms H_2O_2 in water and O_2. Glutathione reductase (GR) is an antioxidant enzyme that maintains the balance GSH/GSSG favouring the reduced form (GSH). GSH is considered a key soluble antioxidant by itself but also as it is a cofactor in several enzymes. As opposed to CAT, GR requires NADPH, and it is therefore a more energy demanding enzyme. Although

not considered in the present study, aquatic organisms possess many other defences either enzymatic (e.g. superoxide dismutase, glutathione peroxidase) or non-enzymatic oxyradicals scavengers (e.g. glutathione, carotenoids, vitamins C and E) that can also play an antioxidant role.

Another hepatic marker selected in our study is part of the cytochrome P450 superfamily. In particular the CYP1A form is a terminal protein in the mixed function oxygenase system, indicative of phase I metabolism (mostly hydroxylation reactions). In fish, its main catalytic role is frequently measured as ethoxyresorufin O-deethylase (EROD) activity and it is a well accepted biomarker of dioxin-like compounds and PAHs exposure. However, diet and some environmental and physiological factors can modulate the response of the total cytochrome P450 content. This is specially evident in the response of the CYP1A dependent EROD activity (Whyte et al., 2000). Carboxylesterases are also classified as phase I enzymes involved in the hydrolysis of many chemicals and have a relevant role in detoxification and cell protection from chemicals action (Satoh and Hosokawa, 2006; Al-Ghais et al., 2000). Foreign chemicals, but also endogenous compounds (e.g. sex hormones), that undergo phase I metabolism (e.g. hydroxylation, oxygenation reactions) can be further conjugated with endogenous molecules, such as GSH, via glutathione S-transferases (GSTs) and consequently, be more readily excreted (phase II metabolism). Moreover, GST activity has also an antioxidant role as peroxidase (George et al., 1994). Both, phase I and II systems are seen to vary seasonally, although variations in GST are much less marked than EROD activity. Protein yield (PY) was adopted as an unspecific marker indicative of hepatic protein synthesis. A ratio between bioactivation (phase I) and detoxification (phase II) named biotransformation index (BTI) has been proposed in fish as indicative of the balance of both paths (van der Oost et al., 1998).

The selection of the fish species was based on their availability and frequency in the catch by the trawling fleet in the selected sites, their economic and ecological interest were also considered. Moreover, the choice of a broad spectrum of species contributes to fill in the gap concerning xenobiotic biotransformation enzymes in marine fish (Fitzsimmons et al., 2007), as most data, particularly in the Mediterranean, refers to the red mullet *Mullus barbatus* and few attempts have been made to use other sentinels. To name a few studies considering species also adopted in this present survey: e.g *Helicolenus dactylopterus* (Amato et al., 2006), *Phycis blennoides* (Garcia de la Parra et al., 2000) and *Merluccius merluccius* (Pietrapiana et al., 2002). The reason for enlarging the number of species, as potential sentinels, and extending the area of study offshore is justified for the interest, in the case of pollution events (*e.g* oil spills, dredging activities, dumping operations), of having previously characterised the natural variations on these hepatic parameters currently applied in pollution monitoring studies. On a previous study, the relationship between xenobiotic metabolism enzymes and fish diet, habitat, phylogeny was pointed out (Solé et al., 2009a). The present study aims to further explore and strengthen, or rebate, these former observations by considering more species (from 9 to 18) as well as by adding temporal (seasonality) and spatial (two sites comparison) variations as novel factors.

2. Materials and methods

2.1. Sampling area and fish collection

The sampling took place seasonally: winter (February), spring (April), summer (July) and autumn (October) in 2007. It was carried out on research (Garcia del Cid-CSIC) and trawling commercial (Stella Maris III) vessels in front of the Barcelona coast (NE Spain) offshore the Besós river on the continental shelf at depths of 50-65 m (41º

24'N, 2º 16'E) and 200 m (41º 20'N, 2º 15'E) and on the continental slope at 600-850 m (41º 12'N, 2º 28'E) and 1000 m (41º 10'N, 2º 30'E). During the summer cruise, fish from another area (Vilanova) were also sampled on the shelf (41º 14'N; 1º 76'E) and slope (41º 07'N; 2 º21'E). Once on board, fish were immediately sexed, weighed, measured and dissected and their livers frozen in liquid nitrogen and transported to the laboratory where they were kept at –80ºC until biochemical analysis. A list of the species selected is given in Table 1. More information, including their phylogeny, common name and depth of occurrence, as well as a map of the selected sites is detailed elsewhere (Solé et al., 2010).

2.2. Physiological markers

Hepatosomatic index (HSI) was calculated as: (liver weight/body weight)x100, condition factor (CF) as: (body weight/length^3)x100 and gonadosomatic index (GSI) as: (gonad weight/body weight)x100. Protein yield (PY) of the liver was calculated as: mg protein/g wet weight.

2.3. Sample preparation and biochemical determinations

A portion of the liver (0.2-0.5 g) was homogenised in phosphate buffer using a polytron® blender and the supernatant (S10) obtained after centrifugation at 10,000g for 30 minutes was used for biochemical analysis. For comparative purposes, assay conditions were kept similar and only the sample volume varied in order to achieve linearity in the enzymatic measurements. All assays were carried out in duplicate or triplicate at 25ºC with the S10 supernatant, with the exception of CAT, CbE and GST activities which required a further dilution. A description of the methodologies for sample preparation and enzymatic determinations: catalase (CAT; EC 1.11.1.6),

glutathione reductase (GR; EC 1.6.4.2), 7-ethoxyresorufin O-deethylase activity (EROD; EC 1.14.14.1), carboxylesterases (CbE; EC 3.1.1.1), glutathione S-transferase (GST; EC 2.5.1.18) as well as total protein content in the S10 fraction was as described elsewhere (Solé et al., 2006; 2009a). Briefly, CAT, GR, CbE and GST were measured spectrophotometrically at the wavelength of 240, 340 (as decreases), 405 and 340 (as linear increases in optical density), respectively, over a one to 5 minutes period using a microplate reader model TECAN Infinite200. EROD was measured fluorimetrically after 30 minutes incubation at 30ºC and using resorufin as standard. Activity was expressed in relation to the total protein measured (Bradford et al., 1976).

2.4. Statistical analysis

Physiogical parameters (Table 1) are presented as mean ± SD and hepatic biomarkers (Table 2) as mean ± SEM. Species differences were tested using parametric one-way (Fig 1) or two-way ANOVA (Table 2) followed by post-hoc test (Newman-Keuls) using the software Statistica v. 7.0. Pairwise comparisons using t-test were applied to site comparisons (Besós and Vilanova). Pearson correlation coefficients were computed to analyse the relationship between pairs of biomarkers.

Canonical Correspondence Analysis (CCA; Legendre and Legendre 1998) was performed with the R library *vegan* to associate biochemical markers with season and quantitative ecological variables in the multidimensional space. CCA is a multivariate technique that allows examining in a reduced dimensional space the relationship between variables (in our case, biomarkers) and linear constraints (in our case, season and ecological variables). The results of the CCA analysis can be visualised through a graph called *biplot* that allows depicting simultaneously the linear constraints and the variables. Significance of the ordination plot was tested through the pseudo-F test

proposed by Legendre and Legendre (1998) with 1000 permutations. The significance of each linear constraint was likewise assessed with the permutational *envfit* routine of the *vegan* package (J. Oksanen, http://cc.oulu.fi/~jarioksa/softhelp/vegan.html).

3. Results

3.1. Ecological and physiological parameters

Biological and ecological features of the fish species analyzed such as size, habitat depth, swimming capacity, trophic level, and the contribution of different faunistic compartments (benthos, suprabenthos and zooplankton) in the fish diet were presented as tables 1 and 2 elsewhere (Solé et al. 2010). Detailed definition of stomach fullness (*F*) as an indicator to estimate the amount of food consumed by fish (indicating the *trophic condition* of fishes) are in Cartes et al. (2008).

Herein we report on physiological parameters such as: condition factor (CF), hepatosomatic (HSI) and, in females the gonadosomatic (GSI) indexes (Table 1). The HSI was seen as species dependent and varied seasonally. The HSI in perciforms (0.5-3.3) was lower than in gadiforms (0.9-6.5) and in the elasmobranchs was the highest (4.8-11.5). On the contrary, the CF (indicator of the nutritional status) was higher in perciforms (0.4-1.3) than in gadiforms (0.2-0.8) and it also displayed seasonality. The GSI was measured in females and tentatively used as indicative of the reproductive period for the species. For shallow species with a full seasonal data set, summer was seen the reproductive period for *M. barbatus* and *T. draco,* whereas in *S. maena* it was winter. In slope species *M. poutassou* and *P. blennoides* a higher GSI corresponded to the autumn period, whereas in *T. scabrus*, *N. aequalis* and *G. melastomus* it was winter.

3.2. Species differences in hepatic markers

Due to unbalanced sampling data sets, to test for species differences on the activities of the hepatic markers: PY, CAT, GR, EROD, CbE and GST, only fish from the winter sampling were selected. This was based on the fact that the winter sampling included to a more complete set of species and did not consider the temporal variations (as a potential confounding factor) due to the observed seasonality in some of the biomarkers (Fig 1; see also below Section 3.3). Nevertheless, *N. aequalis* (summer) and *B. boops* (spring and summer) are also included here for species comparison as no winter samples were available. In addition, in the winter sampling, *M. merluccius* was present at two depth ranges: 50-60 (shelf) and 600-850 m (slope).

A clear decrease in PY content in relation to habitat depth was evidenced (Fig 1A). This relationship was significant and negative between PY and HSI ($r=-0.726$; $p<0.05$; $n=16$), all teleosts groups considered. Antioxidant defences such as CAT (Fig 1B) were, in general, lower in shallow fish (except in *C. linguatula*, *T. minutus* and *M. merluccius*), whereas GR activity (Fig 1C) was species-dependent regardless of habitat. Extreme GST activities (Fig, 1D) were recorded in *T. minutus*, which was 7.5-fold higher than in *C. linguatula* (the species with the lowest GST activity). Phase I EROD (Fig 1E) activity was significantly elevated in the perciforms: *P. acarne, P. bogavareo* and *M. barbatus*. Among slope fish, only *T. scabrus* expressed EROD activity comparable to shallow fish. As for CbE (Fig 1F), *T. scabrus* and *H. dactylopterus* from the slope displayed, by far, the highest activity. Homogeneous groups are indicated by the same letters in the respective figures.

Enzymatic activities considering all the seasons are indicated (Table 2) for each biomarker and species in order to define trends as it enlarged, considerable for some species, the number of individuals considered and thus allowing a more robust statistical analysis.

3.3. Biomarkers in relation to habitat (shelf and slope), group (teleosts and elasmobranchs) and season (winter, spring, summer and autumn)

Two main groups were made: those from the shelf 50-60 m (including *P. bogavareo* from 200 m) and those from the slope (600-850 m) which also considered the most strictly deep-sea *L. lepidion* and *N. aequalis* (1000 m). Shelf fish are mostly represented by perciforms while slope fish are mainly gadiforms and the two elasmobranchs included are each one representative of a habitat: *S. canicula* (shelf) and *G. melastomus* (slope). In Table 2 results for the biomarker responses in relation to habitat are also indicated. Depth influenced all enzymatic activities: CAT, GR and CbE were significantly lower in shelf fish while EROD activity was elevated.

A classification in two main phylogenic groups (teleosts and elasmobranchs) was also made. Clear differences were seen in both groups in all enzymatic activities with the exception of GST activity (Table 2).

Seasonality was also attempted in eleven species (seven from the shelf and five from the slope) as they occurred in, at least, three samplings. All biomarkers varied seasonally although a clear pattern for species and enzymatic activity could not be evidenced. Overall, in the winter sampling, EROD and CbE activities were significantly higher while, in the summer it was CAT (Table 2).

Statistical differences between habitats, groups and seasons are indicated in Table 2 by using different superscripts.

3.4. Site differences

Six species were fished at both sites (Besós and Vilanova) in a sample size big enough (n=10) to allow comparison: *M. merluccius*, *M. barbatus*, *S. maena* from the

shelf and *P. blennoides*, *T. scabrus* and *G. melastomus* from the slope. No site related differences were seen for most biomarkers, with the exception of *P. blennoides* from the Vilanova site, where CAT was significantly inhibited ($F=5.26$, $p=0.034$) while GST and CbE were enhanced ($F=5.99$, $p=0.025$ and $F=13.82$, $p=0.0016$, respectively). However, consideration has to be given to the fish size (34.5 ± 7.8 and 26.5 ± 2.2 cm TL for Besós and Vilanova specimens, respectively). Fish of comparable sizes from both sites, such as *M. barbatus*, exhibited elevated CbE while *T. draco* had reduced GR activity, both species sampled at the Vilanova station.

3.5. Relationship with ecological parameters

Canonical correspondence analysis (CCA) was first attempted with all the species with a complete set of data (14 out of 18) and considering the temporal variation. However, this attempt did not indicate any clear pattern. Thus, we separated fish according to their habitat (shelf and slope) and, in this case, the relationships between biomarkers against season and ecological variables, as explanatory factors, were all significant, explaining 57.4% of the variance in shelf biomarkers and 47.2% in slope biomarkers (fig. 2). Pearson product-moment correlations (r) between individual biomarkers and ecological variables in the shelf and slope are shown in Table 3, which can help assess the strength and direction of correlation between biomarkers and ecological variables shown in the CCA ordination.

CCA analysis from the shelf species (Fig 2A) revealed that swimming was positively related to GR and GST (first axis) and negatively to CAT, while EROD and CbE were positively related to feeding on zooplankton and benthos, respectively, which essentially determined ordination axis 2. Trophic level (TL) was inversely related, in turn, to fullness (F) and suprabenthos feeding. For the slope species (Fig 2B), the

relationship with ecological variables and in particular with the main components of the diet (benthos, suprabenthos, zooplankton) was not as clear as in the shelf group, and also the strength of the ordination was lower, with less than 50% of the data variance explained by the first 2 axes. However, GR and GST were positively related to swimming, as occurred in the shelf, and to feeding on zooplankton, while they were inversely related to feeding on benthos. PY was also positively related to F. Slope species feeding on suprabenthos exhibited higher TL and lower F.

4. Discussion

Transport of pollutants from coastal point sources to greater depths is well documented in the Mediterranean (review of Albaigés et al., 2005). Some chemicals can reach (in some species and for some chemical classes) concentrations closer to those recorded in coastal fish (Escartín and Porte, 1999; Borghi and Porte 2002; Storelli et al., 2004; 2008; 2009). Thus the modulation of xenobiotic metabolising enzymes and antioxidant defences in fish inhabiting these offshore areas is likely to occur. In fact this study evidences that, in general terms, the antioxidant enzymes (CAT and GR), GST and CbE, although not EROD, were more elevated in fish inhabiting greater depths. Depth-related changes in metabolism have largely been studied in pelagic fish but less is known on benthic and benthopelagic species, coincident, the latter group, with the habitat of the fish in this study. Drazen and Seibel (2007) described a decrease in some muscular enzymatic activities in fish in relation to depth, specially for the first 600 m in a study carried out in Pacific waters. This trend being more obvious for pelagic rather than for benthic and benthopelagic fish. Among the reason(s) for the reported lower activities in deeper adapted fish, one is based in the visual-interactions hypothesis and, consequently, would affect those enzymes involved in motility. In addition, most

studies carried out relating fish metabolism and depth are conducted in regions of the world, other than the Mediterranean. In those other regions, changes on environmental parameters (temperature, oxygen concentration) are large and the depths considered elsewhere went up to of 4000 m (Drazen and Seibel, 2007 and references within). In this current study (60-1000 m), a clear decrease in hepatic protein content, indicated as PY (Fig 1A) as well as in muscle protein content (Solé et al., 2010) coincide with observations for other regions and fish species (Childress and Somero, 1979; Janssens et al., 2000, Drazen and Seibel, 2007). Despite this coincidence, in the present Mediterranean study, the physical parameters in water experienced little fluctuations. That is, mean annual temperatures measured at 5 metres above the sea bottom (mab) at both contrasted depths (shelf and slope) within each season was only 1-2ºC apart, except in autumn which was almost 4ºC different. Likewise, oxygen at 5 mab only varied from 0.1 to 0.2 mg/L, as it is characteristic in the Mediterranean. But, what is more relevant for contrasting purposes is the fact that the enzymes here considered refer to those involved in hepatic metabolism rather than to muscular markers, more involved in motility. In short, as a clear decrease in protein over depth did not coincide with a decrease in hepatic activities, xenobiotic metabolism is not likely to be compromised in fish from greater depths.

This study also evidenced great species differences in hepatic markers, regardless of habitat and group. As far as antioxidant parameters concerns, former studies had indicated a decrease in some antioxidant defences: SOD and GPX, although not CAT, in deep sea fish sampled up to 1300 m in Atlantic waters (Janssens et al., 2000). This trend was not seen in this Mediterranean study, as CAT and GR activities were, on the contrary, significantly enhanced in some slope fish including *N. aequalis* and *L. lepidion*. In fact, these results agree with the hypothesis of a metabolically

cheaper antioxidant defence (CAT) in fish inhabiting poorer environments (Janssens et al., 2000). Great species differences are coincident with another Mediterranean study that contrasted antioxidant defences and xenobiotic metabolism in strictly deep-sea fish (1500-1800 m), in which species particularities were attributed to their biology/diet rather than to site trends, as chemical exposure was also similar (Porte et al., 2000). Species differences were also evidenced in *P. blennoides* and the flatfish *Lepidorhombus boscii* sampled in the same NW Mediterranean region and at 350-450 m depth range and, also in this case, fish were exposed to a similar pollution load (García de la Parra et al., 2000). The *a priory* expected site differences in chemical exposure between the two selected sites (Besós and Vilanova) was not confirmed by the hepatic responses, neither by the bile PAH levels (Insausti et al., 2009), nor by the muscular markers or the chemical analysis of the sediment (Solé et al., 2010). Besós site is under the influence of the river with the same name and well-known for its high pollution load (Castells et al., 2008). Vilanova, on the contrary, is a fishing port with a lower industrial influence from the nearby coast. However, it is likely that this port, situated south of the Llobregat river (with an important industrial catchment area), receives the Llobregat river influence as a result of the north-south water currents in the NW Mediterranean. Turbulence events that would enhance pollutants biodisponibility were higher in spring and winter and in the Vilanova site. Neither chemical analysis nor turbulence events support significant Besós-Vilanova site differences or consistent temporal trends, as far as the persistent chemicals analysed concerns (Solé et al., 2010). This also implies that greater pollution gradients are necessary to be reflected in the adopted biomarkers. In fact, field studies to successfully relate biomarker responses to pollution in the Mediterranean, refer to sites closer to land discharges and therefore subject to greater pollution loads (Porte et al., 2000), or linked to particular pollution events: e.g dumping

operations (Regoli et al., 2002; Amato et al., 2006). The levels of PCBs (0.5-5.8 ng/g d.w.), DDTs (1.4-14.4 ng/g d.w.) and PAHs (176-466 ng/g d.w.) encountered in the sediment of the selected sampling sites are in the low range of those reported in sediments from other NW Mediterranean areas (Cardellicchio et al., 2007; Gómez-Gutierrez et al., 2007; Castells et al., 2008).

As it was observed for the antioxidant defences, phase I and II enzymes also evidenced greater species differences rather than site trends. While EROD was particularly enhanced in fish from the shelf, CbE and GST were better represented in slope species. Other studies in the Mediterranean, contrasting deep-sea fish (*L. lepidion* included) with coastal fish also revealed comparable enzymatic activities (Escartín and Porte, 1999). The same trend occurred in an Atlantic study with fish sampled from 300 to 3500 m (Stegeman et al., 2001). Among gadiforms, *T. scabrus* displayed high EROD activity (considering is a slope species), as well as the highest CbE and one of the highest GST activities. *T. scabrus* is one of the species distributed deeper among our targeted ones, and it has a high feeding activity over *Calocaris macandreae*, a burrowing shrimp living into the mud (Cartes et al., 2008). As CbE is a general hepatic metabolism marker, high levels of CbE in *T. sabrus* could be related to higher exposure to sediment bound pollutants in bathyal-muddy bottoms and/or to a particularly high metabolism in this species. On the contrary, the lowest enzymatic defences were clearly displayed by elasmobranchs, confirming earlier observations (Filho et al., 1993a; 1993b, Gorbi et al., 2004, Solé et al., 2009a). Nevertheless, due to their large liver (about 10 g) in the species included in this study, the total hepatic functions in these elasmobranchs might not differ so much from the shelf fish with smaller livers (0.5-2 g). Nevertheless, it has to be taken into account that the liver in elasmobranchs has also an important function in swimming and floatability, thus our observation is merely

speculative. Another important factor to consider when comparing shelf and slope is that all our measurements were carried out under the same assay conditions of substrate concentrations, pH, temperature and pressure while in the natural environment fish will experience particular conditions depending on their habitat that will affect enzymes kinetics and protein interactions, specially in those from greater depths (Gibbs, 1997).

In this study, the ratio EROD/GST termed biotransformation index (BTI) and used as an estimation of phase I/phase II metabolism, was also adopted (van der Oost et al., 1998). While in the biotransformation process EROD can generate chemicals more reactive than the parent compound, GST is always a detoxification step. Due to the larger variations in EROD than in GST activities between species, this index mostly followed EROD trends (Table 2). This BTI ratio could predict that perciforms are more prone to form toxic metabolites, whereas *T. minutus* showed the greatest detoxification capacity. Coincident with *T. scabrus*, *T. minutus* also feeds on prey distributed on or in the mud (e.g. the burrowing shrimp *Alpheus glaber*: authors' unp. data). BTI as an index has been successfully used contrasting sites using a single sentinel species (van der Oost et al 1998), while in the present study several species are contrasted and, the nature of the particular P450s involved, could greatly differ. Nevertheless, this index could be useful as an estimation of an overall biotransformation trend.

Seasonal variation in the hepatic biomarkers was also attempted in this study. Although a common and clear pattern was not observed, enzymatic activities in winter were, in general, higher than in summer (Table 2). Other studies have revealed that the parameter most affected by seasonality is EROD activity, as it is modulated by sex hormones, and this would reflect the reproductive season, mostly in females, by expressing a significantly lower EROD activity (Whyte et al., 2000). An attempt to correlate EROD activity with the GSI in females in two species, one from the shelf, *M.*

barbatus, and one from the slope, *T. scabrus* was made. Nevertheless, EROD activity was not related to GSI as in a mixed group of males, females and immature specimens. When grouping them by sex the sample size was too small to predict any relationships. In future studies more frequent samplings (monthly) and larger sample sizes should be considered in order to test for such correlations.

Due to the large number of species (18), biomarkers (7) and ecological variables (6) considered, a multivariate CCA was carried out in order to relate biomarker responses with ecological parameters in shelf and slope fish. Significant relations were observed (Fig. 2), although some differ from those in a former study (Solé et al., 2009a), despite enzymatic activities in the common species and sampling period were fully coincident. To account for this difference, it could be (1) the fact that fish from shelf and slope are now considered independently, (2) the ecological parameter TL is now given a numeric value and (3) samples from several seasons are included. In this current approach, relationships between biomarkers and ecological variables were all significant ($p<0.001$) although the ecological variables chosen only explained around 50% of the variance observed in the biomarkers. Thus other parameters not considered here are likely to play an important role in the biomarker responses. Overall, our observations confirm the role of seasonality in modulating biomarker responses and the need for adequate references when applying these biomarkers in pollution monitoring. Another important observation extracted from the CCA is that the relationships between hepatic biomarkers and trophic variables were clearer in shelf than in slope fish. This is probably because of the lower prey availability on deeper environments than drives to an increase in the trophic diversification of slope species (Cartes, 1998). That is, slope-dwelling fish exploit, with a similar intensity, prey from different ecological compartments (benthos, zooplankton). In this way, TL was better related to hepatic

biomarkers in shelf than in slope fish. In turn, this coincides with a more simple organization (trophic chain) over the shelf while more complex one (food webs) is present at greater depths. In the slope, in addition to scarcer food there are also more unpredictable pulses of food availability. Moreover, liver in slope fish can also be regarded to act as a storage organ (i.e. liver and HSI in slope fish is bigger than in those from the shelf) and this may differentially affect hepatic enzymes. Even if physical parameters (temperature, oxygen, salinity) at the slope in the Mediterranean remain fairly constant, seasonality is still an important force influencing the fish diet, feeding activity and reproduction (Cartes et al., 2008). On the contrary, in the Mediterranean shelf, significant differences in physical parameters between periods of homogeneity (winter and spring) and stratification (mostly summer) can be encountered due to existence of a thermocline at about 150 m in the Mediterranean (Cartes et al., 2008). Swimming activity also played, as expected, a more marked role in shelf fish. Nevertheless, this role could be enhanced due to the presence in the shelf of *B. boops*, a species which feeds exclusively on pelagic prey while, at the slope, due to above mentioned dietary diversification, an equivalent species was not present (Cartes, 1998).

5. Conclusion

This study evidences great species differences in hepatic biomarkers that contrast with little variations in physical environmental parameters and pollution gradients. Most enzymatic activities, except EROD, were enhanced in slope species (mostly gadiforms) in contrast to shelf species (mostly perciforms), despite pollutant loads in the sediment at both depths being similar. This study further explores relationships between hepatic biomarkers and ecological parameters formerly outlined (Solé et al., 2009a). As the CCA revealed, in the shelf seasonality plays a more

prominent role while, and probably due to the scarcity and unpredictable food supply (among other reasons), in slope fish relationships between hepatic biomarkers and ecological variables are less clear.

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Figure legends

Figure 1. Hepatic biomarkers: A) protein yield, B) catalase, C) glutathione reductase, D) glutathione *S*-transferase, E) ethoxyresorufin *O*-deethylase and F) carboxylesterase from fish species sampled in the winter cruise (except *B. boops* and *N. aequalis*) in the Besós (Barcelona, Spain, NW Mediterranean). Different letters indicate statistical differences ($p<0.05$) using one-way ANOVA.

Figure 2. Results of the Canonical Correspondence analysis (CCA) ordination of hepatic biomarkers against season and ecological variables (trophic level-TL, swimming, fullness-F and type of diet and cruise code). Ecological variables as in Table 1 in Solé et al., (2010). A) shelf fish and B) slope fish. All relations are significant and the ecological variables explain 57.4 % and 47.2 % of the variance for hepatic biomarkers from shelf and slope fish, respectively.

Table 1. Mean and standard deviations of morphological markers: Hepatosomatic Index (HSI), Condition Factor (CF) and Gonadosomatic Index (GSI) in fish species collected at the fishing grounds of Besós. The number of individuals analysed is the same of Table 2 from Solé et al., (2010), except for the GSI in females which is indicated in brackets (n). n.a. not available.

	Winter			**Spring**			**Summer**			**Autumn**		
	HSI	CF	GSI ♀	HSI	CF	GSI ♀	HSI	CF	GSI ♀	HSI	CF	GSI ♀
P. acarne	0.77 ± 0.18	1.25 ± 0.09	n.a.	0.98 ± 0.15	1.19 ± 0.06	n.a.	1.02 ± 0.36	1.19 ± 0.11	n.a.	n.a.	n.a.	n.a.
P. bogaraveo	0.86 ± 0.26	1.20 ± 0.04	0.79 ± 0.23 (2)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
P.erythrinus	1.18 ± 0.28	1.25 ± 0.13	0.20 ± 0.01 (2)	1.41 ± 0.47	1.34 ± 0.09	n.a.	1.36 ± 0.45	1.15 ± 0.25	n.a.	0.94 ± 0.19	1.24 ± 0.07	n.a.
B. boops	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.83 ± 0.13	0.86 ± 0.07	n.a.	n.a.	n.a.	n.a.
M. barbatus	2.59 ± 0.85	1.13 ± 0.11	0.99 ± 0.42 (7)	2.30 ± 0.47	1.02 ± 0.06	0.84 ± 0.08 (3)	3.31 ± 1.02	0.68 ± 0.09	3.32 ± 1.09 (8)	2.05 ± 0.29	1.15 ± 0.07	0.58 ± 0.15 (9)
S. maena	1.90 ± 0.76	1.07 ± 0.12	7.63 ± 1.18 (3)	1.40 ± 0.44	1.01 ± 0.12	2.87 ± 2.27 (5)	0.89 ± 0.14	0.91 ± 0.06	0.44 ± 0.11 (6)	0.27 ± 0.08	0.94 ± 0.17	0.23 ± 0.15 (3)
T. draco	0.50 ± 0.18	0.62 ± 0.03	0.52 ± 0.11 (5)	1.18 ± 0.51	0.64 ± 0.06	0.65 0.40 (7)	1.61 ± 0.63	0.43 ± 0.13	2.13 ± 1.65 (9)	0.70 ± 0.25	0.64 ± 0.06	0.63 ± 0.16 (6)
C. linguatula	1.44 ± 0.75	0.68 ± 0.05	0.57 ± 0.23 (7)	1.03 ± 0.51	0.58 ± 0.13	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
H. dactylopterus	0.94 ± 0.21	1.67 ± 0.18	n.a.	n.a.	1.54 ± 0.14	n.a.	0.96 ± 0.14	1.62 ± 0.14	n.a.	0.92 ± 0.24	1.50 ± 0.10	n.a.
T. minutus	1.84 ± 0.83	0.97 ± 0.13	4.73 ± 0.17 (3)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
M. merluccius	2.69 ± 1.71	0.67 ± 0.12	0.45 ± 0.30 (6)	1.90 ± 0.66	0.33 ± 0.26	n.a.	3.79 ± 1.80	0.64 ± 0.06	0.45 0.17 (5)	2.43 ± 1.10	0.81 ± 0.09	n.a.
M. poutassou	2.66 ± 1.40	0.50 ± 0.11	0.73 ± 0.73 (2)	4.27 ± 1.99	0.66 ± 0.05	0.16 ± 0.05 (7)	6.50 ± 0.81	0.71 ± 0.05	0.21 ± 0.08 (3)	5.52 ± 2.19	0.70 ± 0.04	2.12 ± 1.08 (9)
P. blennoides	1.69 ± 0.70	0.58 ± 0.06	0.37 ± 0.09 (2)	5.51 ± 4.66	0.62 ± 0.20	0.08 ± 0.04 (7)	3.67 ± 1.40	0.72 ± 0.08	0.21 ± 0.20 (5)	4.32 ± 1.68	0.68 ± 0.05	2.47 ± 3.42 (5)
T. scabrus	2.94 ± 0.86	0.32 ± 0.03	1.36 ± 1.28 (4)	3.08 ± 1.27	0.35 ± 0.07	0.57 ± 0.42 (5)	3.09 ± 1.14	0.36 ± 0.02	0.21 ± 0.12 (5)	1.68 ± 0.66	0.37 ± 0.11	0.47 ± 0.22 (7)
N. aequalis	1.51 ± 0.52	0.26 ± 0.04	1.82 ± 0.98 (7)	2.52 ± 1.36	0.24 ± 0.05	1.19 ± 1.35 (5)	2.06 ± 1.56	0.28 ± 0.10	0.45 ± 0.15 (4)	2.53 ± 0.80	0.22 ± 0.04	0.43 ± 0.29 (8)
L. lepidion	2.85 ± 0.95	0.51 ± 0.03	0.23 ± 0.08 (6)	2.56 ± 0.61	0.49 ± 0.03	0.14 ± 0.09 (3)	2.17 ± 0.73	0.55 ± 0.16	0.19 ± 0.09 (5)	n.a.	0.54 ± 0.03	0.21 ± 0.09 (5)
S. canicula	7.19 ± 2.64	0.29 ± 0.03	n.a.	7.42 ± 2.04	0.30 ± 0.07	5.12 ± 1.71 (8)	8.22 ± 1.82	0.37 ± 0.05	3.94 ± 1.40 (5)	6.00 ± 2.27	0.34 ± 0.03	2.96 ± 0.72 (5)
G. melastomus	11.50 ± 4.67	0.21 ± 0.05	1.31 ± 1.73 (3)	7.20 ± 3.12	0.31 ± 0.05	0.32 ± 0.27 (5)	5.86 ± 2.66	0.30 ± 0.03	0.09 ± 0.08 (2)	4.80 ± 1.42	0.29 0.04	0.49 ± 0.53 (6)

Table 2. Biomarker activities considering fish from all the samplings (n). Different letters indicate statistically significant differences ($p<0.05$).

Species (n)	PY[1]	CAT[2]	GR[3]	GST[3]	EROD[4]	CbE[3]	BTI[5]
P. acarne (19)	77.0 ± 3.3^{f}	1048 ± 53^{abc}	12.5 ± 0.7^{bhi}	416 ± 37^{abcd}	115 ± 18^{d}	134 ± 22^{d}	268 ± 36^{b}
P. bogavareo (10)	84.4 ± 7.3^{f}	523 ± 59^{a}	9.9 ± 0.7^{abfgh}	405 ± 26^{abcd}	179 ± 30^{d}	102 ± 8.6^{abcd}	475 ± 87^{c}
P. erythrinus (44)	76.3 ± 2.2^{ef}	832 ± 42^{ab}	7.9 ± 0.5^{afg}	402 ± 19^{abc}	23.4 ± 2.4^{abc}	39.5 ± 4.3^{abc}	56.8 ± 5.1^{a}
B. boops (14)	67.3 ± 2.8^{def}	804 ± 60^{ab}	15.6 ± 1.0^{hi}	281 ± 21^{ab}	13.3 ± 2.8^{ab}	73.0 ± 9.1^{ab}	49.2 ± 10.2^{a}
M. barbatus (50)	71.5 ± 2.2^{cde}	650 ± 44^{abc}	4.6 ± 0.4^{cde}	295 ± 17^{abcd}	29.5 ± 6.6^{bc}	47.5 ± 3.0^{a}	91.9 ± 15.8^{a}
S. maena (39)	67.1 ± 2.5^{bcdef}	642 ± 36^{ab}	10.5 ± 0.5^{ab}	444 ± 28^{abcd}	6.3 ± 0.8^{ab}	55.0 ± 4.2^{abcd}	16.4 ± 2.8^{a}
T. draco (46)	60.9 ± 2.2^{bcde}	557 ± 27^{ab}	6.8 ± 0.5^{efg}	210 ± 11^{abc}	1.7 ± 0.3^{ab}	49.4 ± 4.3^{abcd}	8.0 ± 1.2^{a}
M merluccius (52)	56.8 ± 1.4^{bcd}	2111 ± 154^{bcd}	7.3 ± 0.3^{efg}	336 ± 17.9^{abcd}	19.6 ± 3.3^{abc}	51.6 ± 5.3^{abc}	59.9 ± 10.4^{a}
T. minutus (11)	48.2 ± 2.6^{abcd}	2134 ± 335^{cd}	9.7 ± 1.0^{abfg}	1585 ± 147^{g}	2.7 ± 0.5^{a}	143 ± 17.2^{abcd}	1.7 ± 0.3^{a}
M. poutassou (33)	21.6 ± 2.4^{ab}	1226 ± 103^{ab}	15.4 ± 1.8^{i}	862 ± 94^{abcd}	1.4 ± 0.1^{a}	29.1 ± 2.5^{a}	2.1 ± 0.3^{a}
P. blennoides (41)	35.9 ± 2.1^{abc}	2086 ± 130^{de}	15.0 ± 0.7^{i}	637 ± 50^{abcd}	2.9 ± 0.3^{a}	128 ± 8.2^{cd}	5.3 ± 0.6^{a}
T. scabrus (48)	39.5 ± 1.8^{abc}	3500 ± 293^{d}	9.3 ± 0.7^{abg}	955 ± 77.1^{f}	28.0 ± 4.6^{c}	248 ± 31.4^{f}	33.5 ± 5.6^{a}
N. aequalis (8)	29.1 ± 2.5^{a}	1226 ± 103^{e}	15.4 ± 1.8^{abdefg}	862 ± 94^{ef}	1.4 ± 0.1^{ab}	29.1 ± 2.5^{bcd}	2.1 ± 0.3^{a}
L. lepidion (20)	25.1 ± 1.6^{a}	5236 ± 710^{d}	10.0 ± 1.2^{abg}	791 ± 33^{def}	4.8 ± 1.2^{ab}	68.0 ± 6.6^{abcd}	6.3 ± 1.5^{a}
C. linguatula (10)	53.1 ± 4.0^{bcd}	2280 ± 152^{d}	0.73 ± 0.2^{c}	211 ± 14^{a}	20.2 ± 4.3^{ab}	60.4 ± 4.7^{ab}	98.1 ± 20.4^{a}
H. dactylopterus (41)	60.1 ± 2.5^{bcd}	891 ± 53^{ab}	2.9 ± 0.4^{cd}	359 ± 42^{bcdef}	6.4 ± 0.6^{a}	189 ± 24^{e}	23.9 ± 3.1^{a}
S. canicula (41)	37.5 ± 2.2^{abc}	480 ± 38^{a}	6.2 ± 0.4^{def}	502 ± 30.0^{abcde}	4.5 ± 1.2^{a}	36.7 ± 3.7^{a}	13.3 ± 4.9^{a}
G. melastomus (52)	30.6 ± 1.8^{a}	219 ± 20.4^{a}	5.5 ± 0.3^{cdef}	527 ± 30^{cdef}	2.3 ± 0.2^{a}	17.7 ± 1.3^{a}	5.3 ± 0.7^{a}
Habitat (n)							
Shallow (326)	63.3 ± 1.1^{a}	949 ± 44^{a}	8.0 ± 0.2^{a}	401 ± 15.9^{a}	25.1 ± 8.6^{a}	57.9 ± 2.6^{a}	68.0 ± 6.9^{a}
Slope (243)	36.5 ± 1.2^{b}	1958 ± 134^{b}	9.5 ± 0.5^{b}	681 ± 27.8^{b}	8.6 ± 1.1^{b}	121 ± 9.5^{b}	14.0 ± 1.5^{b}
Group (n)							
Teleosts (476)	55.4 ± 1.0^{a}	1586 ± 75^{a}	9.2 ± 0.3^{a}	521 ± 18.7^{a}	20.9 ± 2.0^{a}	96.7 ± 5.2^{a}	51.8 ± 4.8^{a}
Elasmobranchs (93)	33.7 ± 1.4^{b}	334 ± 24^{b}	5.8 ± 0.3^{b}	516 ± 21.1^{a}	3.3 ± 0.6^{b}	26.1 ± 2.0^{b}	8.9 ± 2.2^{b}
Season (n)							
Winter (151)	52.2 ± 1.8^{b}	1231 ± 74^{a}	9.4 ± 0.4^{b}	610 ± 28.9^{b}	38.5 ± 5.2^{a}	148 ± 13.4^{a}	82.9 ± 12.5^{a}
Spring (126)	59.0 ± 2.0^{a}	960 ± 58.4^{a}	6.9 ± 0.4^{a}	323 ± 15.3^{b}	11.6 ± 1.9^{b}	54.6 ± 3.4^{b}	42.9 ± 7.2^{b}
Summer (133)	47.5 ± 2.0^{b}	1882 ± 199^{a}	9.0 ± 0.4^{b}	504 ± 28.2^{c}	8.5 ± 1.0^{b}	71.8 ± 6.5^{b}	25.6 ± 3.1^{b}
Autumn (79)	45.7 ± 2.8^{b}	1472 ± 230^{a}	8.7 ± 1.0^{b}	722 ± 63.3^{a}	9.9 ± 1.8^{b}	47.4 ± 5.3^{b}	15.7 ± 2.3^{b}

[1] mg prot/g wet weight
[2] ∝mol/min/mg prot
[3] nmol/min/mg prot
[4] pmol/min/mg prot
[5] (EROD/GST) x1000

Table 3. Pair-wise correlations between biomarkers and environmental variables used in the CCA analysis (Fig. 2). Bold type indicates significant correlation at the $p<0.05$ level.

SHELF	Benthos	F	Suprabenthos	swimming	TL.isotopy	Zooplankton
BTI	0.202	-0.075	-0.227	-0.346	0.200	0.184
catalase	-0.261	0.387	0.277	-0.407	-0.088	-0.072
CbE	-0.140	-0.108	0.097	-0.162	-0.159	0.188
EROD	0.241	-0.068	-0.241	-0.238	0.225	0.128
GR	-0.211	-0.221	0.157	0.274	-0.419	0.225
GST	-0.071	0.128	0.142	0.469	-0.055	-0.318
PY	0.449	-0.289	-0.301	-0.181	0.383	0.210
SLOPE						
BTI	0.332	-0.185	-0.069	-0.129	-0.067	-0.264
catalase	0.490	-0.481	0.407	-0.054	0.231	-0.531
CbE	0.541	-0.284	0.017	-0.142	-0.069	-0.466
EROD	0.345	-0.310	-0.118	-0.034	-0.161	-0.263
GR	0.064	-0.269	0.008	0.339	0.027	-0.041
GST	0.105	-0.424	-0.052	0.270	-0.034	-0.069
PY	0.319	0.189	0.027	-0.372	0.016	-0.280

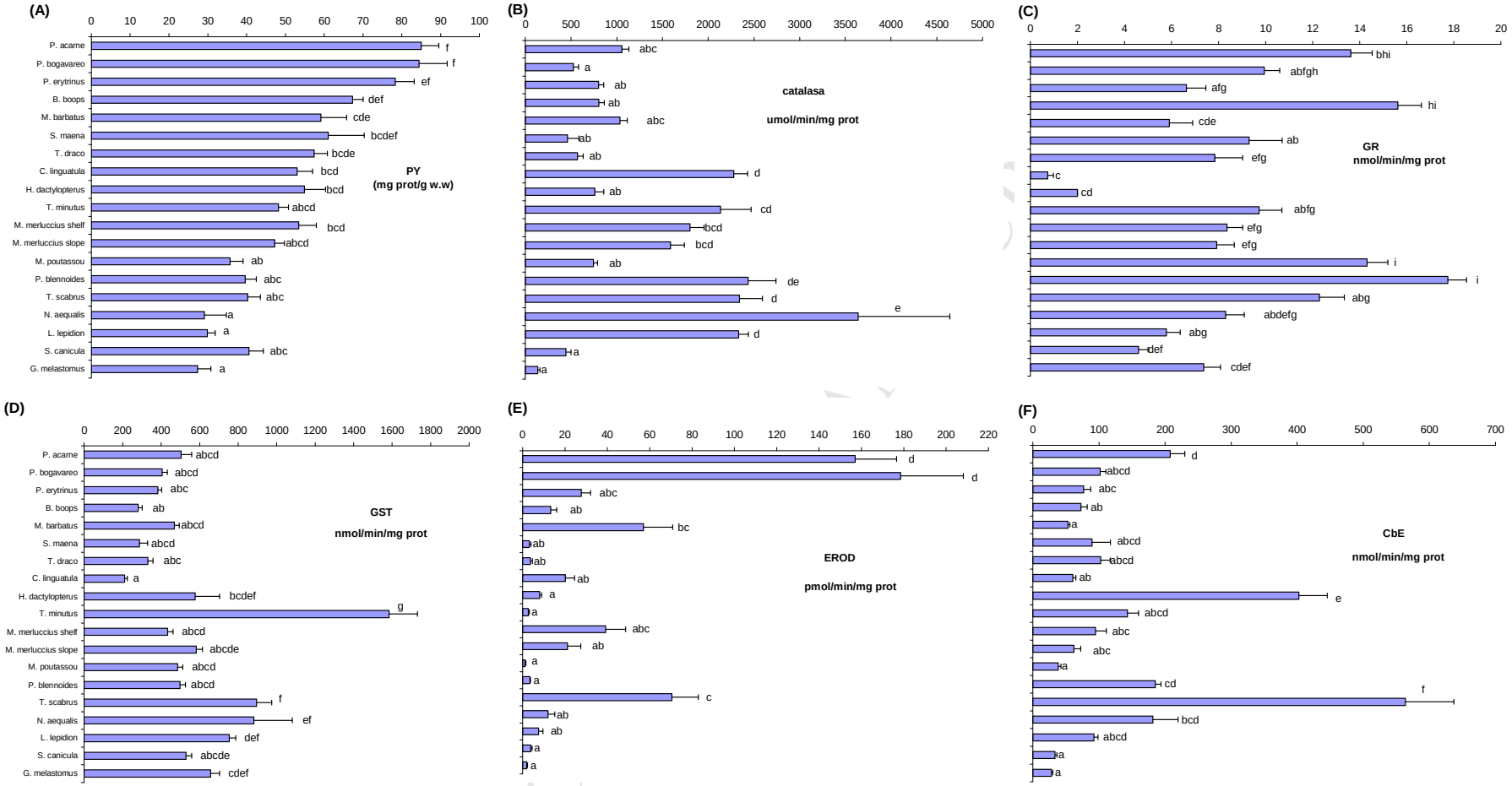

Fig 2A. Shelf

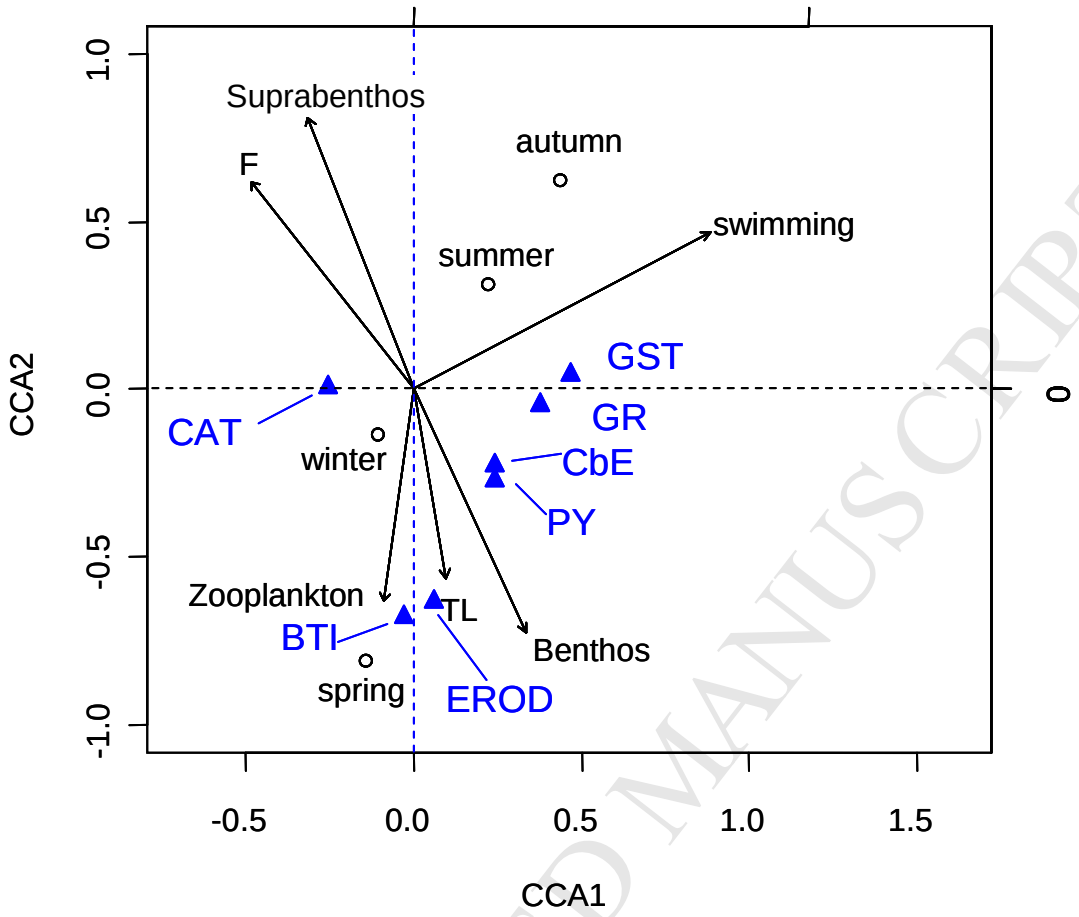

Fig 2B. Slope