Marine Pollution Bulletin 105 (2016) 37–42

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Digestive enzymatic patterns as possible biomarkers of endocrine disruption in the red mullet (*Mullus barbatus*): A preliminary investigation

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ARTICLE INFO

Article history:
Received 22 January 2016
Received in revised form 29 February 2016
Accepted 4 March 2016
Available online 9 March 2016

Keywords:
Digestive enzymes
Endocrine disruptors
Red mullet
Indicators
Northern Sicilian coast

ABSTRACT

During two seasonal trawl surveys (April and October, 2012), red mullet specimens were caught from two sites of the northern Sicilian coast (Western Mediterranean), characterized by different degrees of pollution, to assess whether their digestive enzymes could be cost-effective diagnostic tools for endocrine disruption. Pepsin, chymotrypsin, carboxypeptidases A and B, amylase and lipase were measured in the digestive tract of each fish. During both samplings, significant differences in the digestive enzymatic patterns of fish collected from the two sites were found. In April, pepsin and lipase contents were significantly lower in fish from the most impacted site than in those from the reference site. In October, the enzymatic patterns showed trends different from spring, with controversial results for carboxypeptidases A and B and amylase. Pepsin and lipase patterns suggest a detrimental effect played by organic pollutants and the use of these enzymes as possible biomarkers of exposure to endocrine disruptors.

1. Introduction

Endocrine active substances (EAS), also known as Endocrine Disruptors (EDs), are injurious to predatory birds and fish (Bascietto et al., 1990). Therefore, there is a legitimate concern that low doses of these chemicals in daily diets may be currently impacting human health, causing endocrine disruption (Rivas et al., 2001) and detrimental effects on neurodevelopment (Colborn, 2004). In fish, the biological effects of EDs are generally evaluated through the use of a variety of molecular, biochemical and histological biomarkers, among which some liver and gonadal enzymatic activities (e.g., mixed function oxygenase system, ovarian aromatase) are most common. Several studies have reported on the effects of organic pollutants of different chemical nature on the activity of digestive enzymes in fish (see Filippov et al., 2013 for a review); a decrease has generally been observed in digestive functionality after exposure to contaminants during "in vitro" experiments simulating chronic pollution, suggesting that the activity of digestive enzymes could be used as potential biomarkers in aquatic toxicology. However, field studies supporting this hypothesis are lacking. To assess whether and at what extent digestive enzymes respond to environmental contamination in the natural environment, a preliminary study has been performed on red mullet (*Mullus barbatus*, Osteichtyes, Perciformes), in the framework of the coordinated research project "Food and environmental safety: the problem of endocrine disruptors", funded by the Italian Ministry of Health. The red mullet is a marine fish commonly found on gravel, sand and mud bottoms at a depth range of 10–270 m (Lombarte et al., 2000), which is recommended as a sentinel species in environmental monitoring programmes (UNEP/RAMOGE, 1999; Lionetto et al., 2003; Martin-Skilton et al., 2006; Zorita et al., 2008; Law et al., 2010), because of wide geographical distribution, non-migratory behaviour, and feeding habits. In fact, it feeds mainly on organisms living in close association with sediments, where most contaminants accumulate, which favours xenobiotic accumulation (Regoli et al., 2002; Esposito et al., 2014). The individuals of red mullet analysed in the present research were caught from two sites at different anthropogenic impact in western Mediterranean, one of which (Milazzo) has been recognized as a pollution hot spot by the Strategic Action Programme (SAP) of UNEP (UNEP/WHO, 2003; EEA, 2006), being subjected to contamination by polycyclic aromatic hydrocarbons (PAHs), heavy metals, and organochlorinated compounds (Caruso et al., 2004; Yakimov et al., 2005; ARPA Sicilia, 2008; Fasulo et al., 2010). This study reports preliminary data on a set of enzymatic activities measured in the gastrointestinal tract of red mullet, which suggest altered digestive capacity in fish from the most impacted site.

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http://dx.doi.org/10.1016/j.marpolbul.2016.03.005
0025-326X/

2. Materials and methods

2.1. Sample collection and treatment

Specimens of red mullet were captured during two seasonal trawl surveys (April and October, 2012), from two different sites along the northern Sicilian coast (Western Mediterranean), characterized by different degrees of pollution: the first site (thereafter indicated as Impact site) was located in a dense shipping traffic area, close to city and harbour of Milazzo, in front of an oil refinery and a thermal power plant; these anthropogenic factors have been demonstrated to affect significantly the marine environment of the harbour (Yakimov et al., 2005; Fasulo et al., 2010). The second site (thereafter indicated as Control site), was located in the Gulf of Patti, a leisure area interdicted to both industrial activity and commercial fishing (Fig. 1).

The whole digestive tract — including the chyme content — of each individual (n = 10 per site) was removed and then divided into different organs (stomach, pyloric caeca and intestine), which were stored at −20 °C until enzyme analysis. After homogenization in buffer 50 mM of Tris HCl pH 7.0 and centrifugation at 3000 rpm × 20 min at +4 °C, the supernatant obtained was used as crude enzymatic extract for measurements of the pepsin, chymotrypsin, carboxypeptidases A and B, amylase and lipase, according to the analytical procedures reported below.

2.2. Enzymatic activity assays

Peptic activity was measured using the method of Anson (1938) modified according to Rick (1974a). The reaction mixture, consisting of 0.5 ml of bovine hemoglobin (2% in 0.06 N HCl; pH 2.00) as the substrate and 0.1 ml of the enzyme extract, was incubated for 10 min at 35.5 °C, then the reaction was stopped by addition of 1.0 ml of 5% trichloroacetic acid followed by centrifugation at 3500 rpm for 10 min. A control tube consisted of the same reagents, but the enzyme extract was added after incubation. Absorbance of supernatant was measured at 280 nm and L-tyrosine was used for calibration. Pepsin activity was determined as 1 Unit = micrograms of tyrosine released per minute from the substrate.

Trypsin activity was determined using the method of Hummel (1959) modified by Rick (1974b), with p-toluenesulfonyl-L-arginine methyl ester (TAME) as the substrate. The reaction started with the addition of enzyme extract (0.05 ml) to a mixture of 0.15 ml of 10.0 mM TAME solution and 1.3 ml of 46.0 mM Tris buffer at pH 8.10 with 11.5 mM $CaCl_2$ buffer. The change in absorbance was measured at 247 nm during 3 min at 25.0 °C. Trypsin activity was reported as 1 Unit = 0.001 absorbance increase per minute.

Chymotrypsin activity was determined using the method of Hummel (1959) modified by Rick (1974c), with N-benzoyl-L-tyrosine ethyl ester (BTEE) as the substrate. The reaction started with the addition of enzyme extract (0.05 ml) to a mixture of 0.70 ml of BTEE (80.0 mM in 50% methanol) and 0.75 ml of 80.0 mM Tris buffer at pH 7.80 with 0.1 M $CaCl_2$ buffer. The change in absorbance was measured at 256 nm during 3 min at 25.0 °C. Chymotrypsin activity was reported as 1 Unit = 0.001 absorbance increase per minute.

Carboxypeptidases A and B were measured using Hyppuryl-L-arginine and Hyppuril-L-phenylalanine as the substrates, respectively (Appel, 1974). For carboxypeptidase A, 0.15 ml of enzyme extract were mixed with 1.35 ml of 1.1 mM hyppuryl-L-phenyalalanine in 27.5 mM Tris buffer 0.11 M NaCl at pH 7.60. For carboxypeptidase B, 0.15 ml of enzyme extract were mixed with 1.35 ml of 1.1 mM hyppuryl-L-arginine in 27.5 mM Tris buffer 0.11 M NaCl at pH 7.60. The change in absorbance was measured at 254 nm during 3 min at 25.0 °C. Carboxypeptidases A and B were reported as 1 Unit = 0.001 absorbance increase per minute.

Amylase activity was determined using the method of Bernfeld (1955) modified by Rick (1974d). The reaction mixture, containing 0.05 ml of enzyme extract, 1.0 ml solution of soluble starch (1% in phosphate buffer pH 6.9) as the substrate and 1.0 ml of 20 mM phosphate buffer at pH 6.9 with 10 mM NaCl, was first incubated for 10 min at 25.0 °C. After addition of Dinitrosalicylate (DNS) reagent (2.0 ml of 1% DNS, 30% sodium potassium tartrate), the mixture was placed in boiling water for 5 min and cooled at room temperature for 30 min. Absorbance of the mixture was determined at 540 nm, using maltose for calibration. Alpha-amylase activity was reported as 1 Unit = 1 μg of maltose released per minute.

Lipase activity was determined using the method of Tietz and Fiereck (1966). The reaction mixture, containing 0.5 ml of enzyme extract, 5.0 ml of olive oil emulsion as the substrate (prepared with 0.2 g of sodium benzoate, 7.0 g of arabic gum, 100 ml of demineralised water and 100 ml of olive oil) and 1.25 ml of 0.2 M Tris buffer at pH 8.0, was incubated for 2 h at 37.0 °C. After addition of 1.5 ml of

Fig. 1. Sampling areas located in Sicily (Italy) along the Tyrrhenian coast: Milazzo (Latitude 38°12′73″N, Longitude 15°17′92″E Average Depth 31 m); Mongiove-Patti (Latitude 38°09′77″N, Longitude 15°00′03″E, Average Depth 53 m).

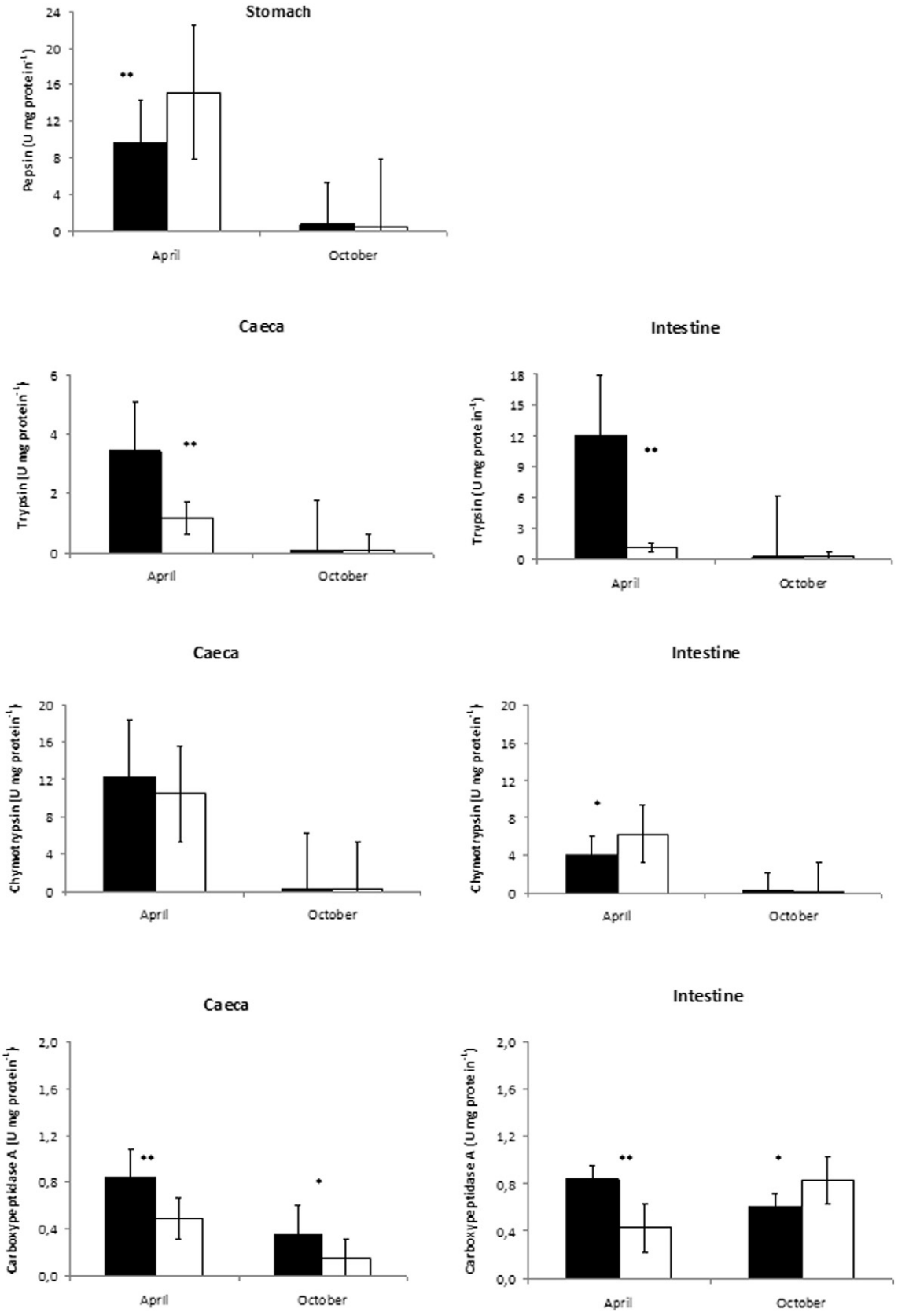

Fig. 2. Mean values ± standard deviation of digestive enzymes found in different organs of red mullet (n = 10 individuals). The asterisks indicate that statistical differences between Impact and Control site occurred at a probability level of $P < 0.01$ (**) and $P < 0.05$ (*).

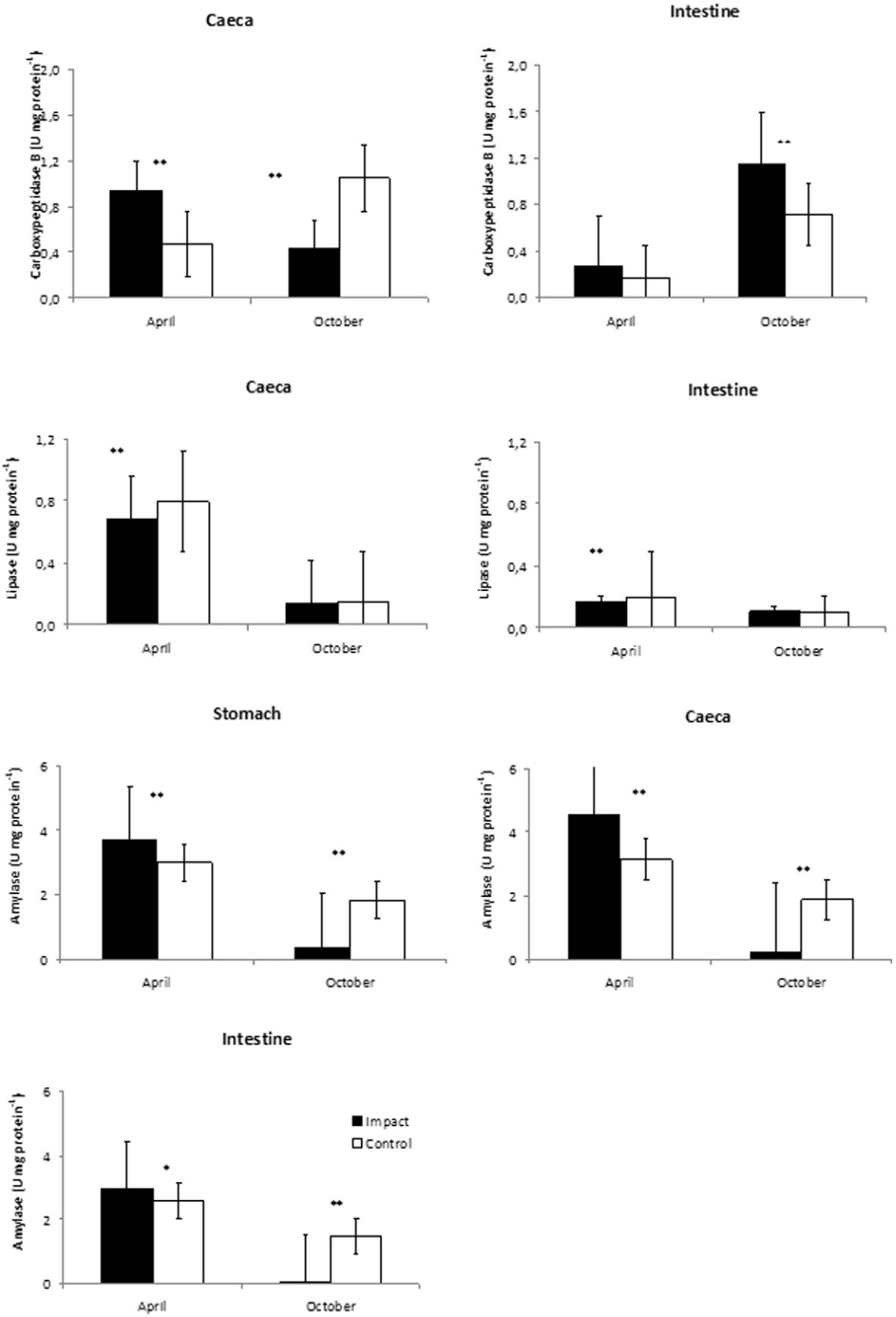

Fig. 2 (*continued*).

95% ethanol, the fatty acids released from the olive oil substrate were titrated with 0.05 N NaOH. Lipase activity was reported as 1 Unit = 1 μl of NaOH.

The measured enzymatic values were normalized to the protein content, determined according to the Lowry et al. (1951), and reported as specific activities (units of enzyme mg protein^{-1}).

2.3. Statistical analyses

Homogeneity of variances and normality tests were performed on digestive enzyme activity values. One-way analysis of variance (ANOVA) followed by Student–Newman–Keuls (SNK) comparisons was applied to assess the significance of differences in the enzymatic values related to sampling site. Differences were statistically significant when Probability was $P < 0.05$. The Sigma-Stat statistical programme version 3.0 was used.

3. Results and discussion

Specific enzyme activity values obtained from the examined samples are reported in Fig. 2.

During both samplings, significant differences in the digestive enzymatic patterns of red mullets collected from the two sites were found. In April, the concentrations of pepsin, intestinal chymotrypsin and lipase were significantly lower in fish from the Impact site compared to those collected from the Control site, whereas trypsin, carboxypeptidase A, caecal carboxypeptidase B and amylase were higher. In October, enzymatic activities were generally lower than in April. Fish from the Impact site showed significant increases in intestinal carboxypeptidase B and caecal carboxypeptidase A, whereas significant decreases in amylase, caecal carboxypeptidase B and intestinal carboxypeptidase A were apparent, compared to fish from the Control site. Overall, pepsin and lipase patterns were suggestive of a detrimental effect played by anthropogenic pollutants, while carboxypeptidases A and B and amylase patterns appeared controversial during the two samplings.

To our knowledge, this is the first study dealing with the effects induced in digestive enzyme patterns by possible exposure to EDs in red mullet. In the same fish species, variations in the levels of acetylcholinesterase and antioxidant enzymes (catalase and glutathione peroxidase) were assayed as potential biomarkers for chemical pollutant exposure (Lionetto et al., 2003), while Martin-Skilton et al. (2006) provided evidence of endocrine disruption through measurements of key enzymatic activities involved in the synthesis and metabolism of steroids, such as ovarian 17 beta-hydroxysteroid dehydrogenases and P450 aromatase.

Studies on the environmental quality of the sediments of the Gulf of Milazzo were focused on the concentrations of PAHs, PCBs and heavy metals and on ecotoxicological effects on marine fauna (Yakimov et al., 2005; ARPA Sicilia, 2008; Fasulo et al., 2010). A first analysis of sediment samples from the Milazzo harbour (Yakimov et al., 2005) evidenced the presence of five major classes of organic (hydrocarbon) pollutants, clearly originating from human activities, among which fatty acids methyl esters (C11-C17 atoms), alkanes (C12-C16 atoms) and alkyl phthalates reached concentrations of 722, 143 and 61 μg/kg of sediment (dry weight), respectively. The origin of these compounds was attributed to the oil hydrocarbon wastes probably discharged from the petrochemical refining plants located in the harbour; alkyl phthalates are used as plasticizers for many polymers. A further report produced by ARPA Sicilia (2008) showed concentrations of benzofluoranthrene and benzopyrene of 861.9 and 683.3 μg/kg of sediment respectively (exceeding the threshold limits of 500 and 400 μg/kg of sediment reported by the Legislative Decree 152/06 in force for environmental protection). Among total PAHs, fluoroanthrene, benzoanthracene, and anthracene were found by Fasulo et al. (2010) at high levels (605.3, 528.7 and 56.5 μg/kg of sediment), which always exceeded the threshold limits. Heavy metal contamination was also present, due to lead concentrations of 219.1 μg/kg of sediment, while PCBs were lower than the prescribed values. Phenanthrene, fluoranthene and pyrene are human-produced pollutants of pyrolytic origin and their recovery is very frequent in the Mediterranean Sea, explaining their presence also in sites that apparently seem unpolluted. The PAHs present in the examined sediments are recognized to be very dangerous for human health (Health Ministry Decree n. 367 of 06/11/2003). Apart from the ranges of PAHs retrieved from literature, the enzymatic results obtained in the present study confirm that red mullets collected from sites affected by different levels of contamination exhibit differences in their digestive metabolism. Particularly, carboxypeptidases A and B and amylase during the two samplings yielded controversial results, so discouraging their possible use as biomarkers of EDs impact on fish digestive functionality; conversely, lipase, pepsin and trypsin seemed to give comparatively more consistent responses, depicting a decreasing effect for pepsin and lipase and a stimulating effect for trypsin. Digestion of dietary fat and protein results in activation of different endocrine pathways which regulate pancreatic secretion. Cholecystokinin (CCK) and peptide Y (PY) play antagonistic roles in the secretion of digestive enzymes, including lipase, with CCK stimulating while PY inhibiting enzyme secretion from the exocrine pancreas (Murashita et al., 2007). CCK and PY are secreted by endocrine cells located within intestinal mucosa, in response to fat or protein ingested with food. Further studies are needed to confirm that such cells might be the target for EDs.

The altered digestive capacity — at least for pepsin and lipase — observed in fish from the Impact site represents the most interesting feature of the present research, as most of the studies concerning the effects of EDs generally focus on reproduction and reproductive behaviour. Conversely, research on the possible effects of EDs on functions other than reproduction may help in identifying specific targets for endocrine disruption (Porte et al., 2006; Casanova-Nakayama et al., 2011; Dimastrogiovanni et al., 2015). EDs may have a critical influence on fish metabolism (Migliarini et al., 2011). On the other hand, digestive enzyme patterns give indication of how active is fish metabolism in digestion. Fish capability to metabolize a diet depends on the availability of appropriate digestive enzymes, which mediate specific degradation pathways, as well as on both physical and chemical nature of food. Investigations regarding the nature and distribution of specific activities (proteases, carbohydrases and lipases) along the gastrointestinal tract of Teleosts may give information on the whole digestive capacity and the efficiency of species reared to use feeding components (Caruso et al., 2009), allowing also to estimate changes in the metabolism in response to xenobiotic compounds acting as ED.

Amiard-Triquet et al. (2012) underlined that digestive enzymes could be relevant physiological parameters to evaluate the effects — or at least the exposure — of organisms to pollutants in laboratory conditions or in situ and consequent implications for energy metabolism as well as vital functions such as growth and reproduction. Compared to conventional measurements of the effects of disturbances at individual level (growth, condition index, reproduction), digestive enzymes show a prompt response and this could be an important advantage for their use as potential biomarkers in ecotoxicology. The decreasing trend detected for pepsin and lipase during April suggests a detrimental effect played by EDs on protein and lipid metabolism, offering a new perspective for the use of these enzymes as possible biomarkers of exposure to organic pollutants.

4. Conclusions

Further investigations are needed to draw solid conclusions on the ED effects on digestive enzymes and assess whether these compounds can target fish digestive processes; particularly, further studies must clarify the mechanisms through which contaminants affect the activity or secretion of digestive enzymes, as well as to determine any possible interferences from abiotic and biotic factors (i.e. temperature, salinity, age, sex, reproductive cycle). Nevertheless, the use of organisms and of specific biomarkers such as lipases as bioindicators could allow to obtain information on the exposure to contaminants and indirectly to environmental health that could not be otherwise possible considering the chemical analysis of abiotic matrices only.

Acknowledgments

This study was supported by the Italian Ministry of Health within the Project "Food and environmental safety: the problem of endocrine disruptors", grant number RF-2009-1536185.

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