HISTOLOGICAL BIOMARKERS IN FISH AS A TOOL IN ECOLOGICAL RISK ASSESSMENT AND MONITORING PROGRAMS: A REVIEW

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Abstract. Water contamination, both in freshwater and marine ecosystems, has been a serious environmental problem all over the world in the last few decades. One of the most common anthropogenic pollutants, which enter the water bodies are metals and metalloids with no biological functions (As, Cd, Hg, Ni and Pb), pesticides and other persistent organic pollutants such as PAHs, PCBs, tributil tins, dioxins, etc. Most of these pollutants tend to accumulate in biota, biomagnify in the food chains and they are also difficult to break down to less harmful substances. In order to better understand the negative effects on living organisms, and particularly fish, biomarkers at different levels (cell, tissue, organism and population) are applied. In addition, the biomarkers at tissue level such as histological alterations in different fish organs give valuable information about the xenobiotic impact. Thus, they are recommended as useful biomarkers in eco-toxicological research, risk assessment and monitoring programs. In the present paper we aimed to review the use of histological alterations in fish organs such as gills, liver and kidney in ecotoxicological studies, based on collected scientific data from the late 1960's until today.
Keywords: *aquatic contamination, fish, biomarkers, histological alterations*

Introduction

According to López-Barea (1995) human activities induce stress on ecological systems by importing pollutants, modifying habitats, introducing exotic species or removing the native ones and by changing climate. These activities not only affect the survival or the performance of individual organisms, but also the structure and function of natural ecosystems and the diversity of life at several levels of organization, including the number of species, the genetic composition, and the variety of ecosystems and landscapes.

It has been reported that in recent decades the level of foreign compounds in aquatic ecosystems such as heavy metals, pesticides and other persistent organic pollutants has increased alarmingly as a result of domestic, industrial and agricultural effluents (Sekabira et al., 2010; Lushchak, 2011; Mohamaddi et al., 2011; Ondarza et al., 2010, 2011; Pereira et al., 2013; Jörundsdóttir et al., 2014). Contamination of aquatic systems is treating the living organisms and thus, it has attracted the great attention of many researchers around the globe (Dutta and Dalal, 2008). Recently, the Water Framework Directive (WFD) of the European Union specified monitoring programs required to assess the achievement of good chemical and ecological status for all water bodies by 2015 (Sanchez and Porcher, 2009).

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Why use fish in eco-toxicological research?

According to Water Framework Directive (WFD), fish represent one of the key elements to evaluate the rivers ecological status (Scardi et al., 2008; Hermoso et al., 2010). Fish have also been found to be good indicators of water contamination in aquatic systems because they occupy different trophic levels; they are of different sizes and ages and in comparison with invertebrates, are also more sensitive to many toxicants (Powers, 1989; Barack and Mason, 1990 a,b; Wenderlaar Bonga and Lock, 1992; Wester et al., 1994; Burger et al., 2002). Fish respond to environmental toxic changes with adapting of their metabolite functions (Mishra and Shukla, 2003). They are present virtually in all environments and many species have been found to be susceptible to environmental pollutants (van der Oost et al., 2003). According to de La Torre et al. (2005) monitoring sentinel fish species is widely used to assess the degree of toxicant accumulation and their effects on health status. Fish are also preferred in toxicological research because of their well-developed osmoregulatory, endocrine, nervous, and immune systems compared to invertebrates (Song et al., 2012). In addition, fish may absorb toxicants directly from the surrounding water and sediments (waterborne exposure), or ingest them through contaminated food in the food chain (dietary exposure), enabling the assessment of contaminant transfer through the tropic web (Cid et al., 2001; Fisk et al., 2001; Moiseenko and Kudryavtseva, 2001; Rashed, 2001; Mondon et al., 2001; Mansour and Sidky, 2002; Usero et al., 2004; Mendil and Uluözlü, 2007; Öztürk et al., 2010; Sounderajan et al., 2010). In teleost fish, the gills, liver, kidney and muscles are the tissues most frequently utilized in ecological, toxicological and pathological studies (Sauer and Watabe, 1989; Velcheva, 2006; Heier, et al. 2009) because they are metabolically active tissues and tend to accumulate toxicants at higher levels (Andres et al., 2000; Karadede and Unlu, 2000; Marcovecchio, 2004). Terra et al. (2008) consider that the toxicants enter the fish organism mainly through the gills and consequently, with the blood they reach the parenchymal organs where they retain for a longer time. In addition, according to Kroglund et al. (2008) the toxicant concentrations, particularly in gills reflect the toxicant concentrations in the water where the fish live; whereas, the concentrations in other organs such as liver and kidney represent storage of toxicants.

Biomarkers in eco-toxicological research

According to van Der Oost et al. (2003); Giari et al. (2008) and Maria et al. (2009) connections must be established between external levels of exposure, internal levels of tissue contamination and early adverse effects and determining the extent and severity of such contamination only by the result of water chemical analysis is insufficient and often overestimates the proportion and duration of exposure to the toxic agent. In the past 25 years, numerous biomarkers have been developed with the objective to apply them for environmental biomonitoring and risk assessment programs (NRC, 1987; McCarthy and Shugart, 1990; Hinton et al., 1992; Peakall, 1992; Hinton, 1994; Bucheli and Fent, 1995; Van Gestel and Van Brummelen, 1996; Shugart and Theodorakis, 1998; Cajaraville et al., 2000; Adams et al., 2001; Triebeskorn et al., 2002; Pandey et al., 2003; Ronisz et al., 2004; Weeb et al., 2005; Martin-Diaz et al., 2006; Schmitt et al., 2007; Martin-Diaz et al., 2008; Álvarez-Muñoz et al., 2009; Weeb and Gangon, 2009; Domingues et al., 2010; Souza et al., 2013; Muñoz et al., 2015). Biomarkers are defined as responses to any exposure evidenced in histological, physiological, biochemical,

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genetic and behavioral modification (Fossi and Marsili, 1997; Fossi, 1998). More recent, van der Oost et al. (2003) defined biomarkers as biological indicators from an exposure to a stressor responding in various ways. Picado et al. (2008) add that biomarkers, which act as early warning signals of the presence of potentially toxic xenobiotics, are useful tools for assessing either exposure to, or the effects of these compounds providing information about the toxicant bioavailability. Pinto et al. (2009) and Viana et al. (2013) suggest that the biomonitoring process should include analyses at different levels of biological organization, from sub-cellular and cellular analysis of tissues and organs, to the of population and community levels. Therefore, studies using biomarkers are essential to complement environmental monitoring in order to control pollution effects on the animals that inhabit the water bodies (Au, 2004; Camargo and Martinez, 2006; Cazenave et al., 2009). In fact, it has been argued that a full understanding of ecotoxicological processes must consider an integrated multi-level approach, in which molecular impact is related with higher-order biological consequences at the individual, population and community levels (Picado et al., 2008). According to NRC (1987); Schlenk (1999) and WHO (1993) biomarkers have been classified into three separate categories that correspond to three major parameters necessary to conduct ecological risk assessments. To perform such an accurate ecological risk assessment, ecological effects, as well as exposure and susceptibility to contaminants must be well-characterized following identification or formulation of a problem. In each of these processes, well-defined biological indicators can be used in certain cases to help make inexpensive predictions regarding the bioavailability (exposure), mechanism of action (effect) and uncertainty of response (susceptibility) elicited by various anthropogenic substances (Schlenk 1999).

Histological alterations as biomarkers

Histopathology involves the microscopic examination of cells and tissues of an organism and the semi-quantitative determination of histological abnormalities (Yevich and Yevich, 1994; The et al., 1999; Peebua et al., 2006, Costa et al., 2010). Greenfield et al. (2008) and Poleksić et al. (2010) explain that the histological alterations in selected target organs are sensitive biomarkers for xenobiotic effects, they occur earlier and provide a better evaluation of the effects of aquatic pollution than any single biochemical parameter. Therefore, analysis of histological changes in different fish tissues has been widely used for decades as an instrument in aquatic toxicology to monitor acute and chronic situations, and to provide additional information to physicochemical analyses (Wester and Canton, 1986; Wester and Canton, 1991; Johnson et al., 1993; Perry and Laurent, 1993; Schwaiger et al., 1997; Stentiford et al., 2003; Schwaiger et al., 2004; Lang et al., 2006; Nero et al., 2006; Monteiro et al., 2008; van Dyk and Pieterse, 2008; Olarinmoye et al., 2009; van Dyk et al., 2009; Liu et al., 2010; Sousa et al., 2013a, b). In addition, several biomonitoring programs have used the histological changes observed in different fish organs as biomarkers for ecological quality of aquatic ecosystems (Lang et al., 2006; Blazer et al., 2007; Pinto et al., 2009). Braunbeck et al. (1990) and Schwaiger et al. (1997) state that the histopathological alterations can be used as indicators for the effects of various anthropogenic pollutants and they are a reflection of the overall health of the entire population in the studied ecosystem. Furthermore, histology is a sensitive tool for the diagnosis of direct and indirect toxic effects that affect the animal tissues (Braunbeck and Volk, 1993; Bernet et

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al., 1999; Poleksić et al., 1999; Ferreira et al., 2005; Marchand et al., 2009; Fanta et al., 2003; van der Oost et al., 2003; Zimmerli et al., 2007, van Dyk et al., 2009; Grund et al., 2010; Rajeshkumar and Munuswamy, 2011; Paithane et al., 2012). Therefore, it is considered as an excellent method for assessing the environmental quality (Costa et al., 2009). In addition, according to Hinton and Lauren (1990) for field assessments, histopathology is often the easiest method of assessing, both short and long-term toxic effects. On the other hand, Wester and Canton (1991) and Yevich and Yevich (1994) state that the histological methods are relatively labor-intensive and require some experience, but after all they have the considerable advantage that pathological alterations in different tissues (e.g., gills, liver) can be observed individually, thus creating a direct link with physiological functions such as growth, reproduction, respiration and nutrition.

The feasibility of using histopathological parameters in fish as a biomarker for aquatic pollution in marine biomonitoring has been reviewed thoroughly by Au (2004). According to Rabitto et al. (2005) and Oliveira Ribeiro et al. (2006) the exposure of fish to chemical contaminants can induce a number of lesions and injuries to different organs, but the gills and liver represent important target organs suitable for histopathological examination in searching for damages to tissues and cells.

Histological alterations in fish gills

Fish gills are multifunctional organs involved in ion transport, gas exchange, acid–base regulation and waste excretion (Dang et al., 2001; Gernhöfer et al., 2001; Evans et al., 2005; Oliveira Ribeiro et al., 2005; de La Torre et al., 2005; Vigliano et al., 2006; Nigro et al., 2006; Salamat and Zarie 2012; Singh, 2014). According to Playle (1998) given the fact that the gills account for over 50% of the surface area of a fish, it is not surprising that one of the major target organs for waterborne toxicants are exactly the gills. Furthermore, according to Carpene and Vašak (1989); Perry and Laurent (1993); Pourang (1995); Tkacheva et al. (2004) and Rosseland et al. (2007) the gills are the main route of toxicants penetration into the fish organism, thus they are the first organs, which come in contact with environmental pollutants and are sensitive subjects for identifying the effects of water toxicants on the fish organism. Thus, the fish metabolism, acting principally through the gills can be seriously damaged since toxicant incorporation occurs mainly through this respiratory organ (Bervoets and Blust, 2003; Sloman, 2007; Terra et al., 2008). Heier et al. (2009) state that as the fish gills can accumulate bioavailable pollutants, their measurement on gills can reflect the speciation of pollutants, and in particular metals in water, which make them a useful tool for assessing metal bioavailability in water. All this helps to understand why in teleost fish the gills are the most frequently utilized in bioaccumulation studies and the pathological damage produced allows defining the toxicity of the environment, making fish highly suitable for evaluating the health of aquatic systems (Mallatt, 1985; Oliveira Riberio et al., 2000; Olsvik et al., 2001; Moiseenko et al., 2005; Ogundiran et al., 2009). The fish gills are also very sensitive to physical and chemical alterations of the aquatic medium such as: temperature, acidification of the water supply due to acid rain, salts and heavy metals, and to any change in the composition of the environment, which is an important indicator of waterborne toxicants (Saber, 2011). Moreover, the gill surface serves as metal-binding ligands and metal bioaccumulation in particular can occur due to positively charged metal species in the water to negatively charged sites on the gills (Playle et al., 1993; Teien et al., 2006 a,b).

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The normal gill morphology of most teleost fish species is described in details by Laurent and Perry (1991); Perry (1997) and Wilson and Laurent (2002). They explain that the gills of most teleost fish are typically composed of four pairs of gill arches, which are supported by a bone skeleton. The gill itself is made up of double rows of filaments, from which arise perpendicularly the lamellae. The filaments come from the gills arches, supported by cartilage (primary lamellae), from which the secondary lamellae exit. The primary lamellae are lined by a squamous epithelium composed by pavement and non differentiated cells. Below that epithelium are lamellar blood sinuses separated by pillar cells. Between the lamellae, the filament is lined by a thick stratified epithelium constituted by several cellular types, such as chloride, mucous and pavement cells. The secondary lamellae are constituted by a simple epithelium where gas exchanges occur. When it comes to biomarkers the gill histological alterations are considered as non-specific biomarkers, which means that many different organic and inorganic contaminants can cause tissue changes. However, they are recognized as a valid and fast method to determine the damage caused in fish by the pollutant exposure (Arellano et al., 1999). Therefore, the gill histological changes have been suggested as useful biomarkers of environmental contamination (Au, 2004; Fernandes et al., 2007; Flores-Lopes and Thomaz, 2011; Adeogun, 2012; Pereira et al., 2013). There are reports on various histological changes in fish gills from contaminated water, both in field and laboratory conditions after acute or chronic exposure with sublethal toxicant concentrations (Doughtie and Rao, 1983; Lauren and Mcdonald, 1985; Alazemi et al., 1996; Mazon et al., 2002; Rao et al., 2003, 2006; Camargo and Martinez, 2007; Matos et al., 2007; Liu et al., 2010; Velcheva et al., 2010 a,b; Muthukumarave et al., 2013; Sousa et al., 2013 a, b), but it is often difficult to decide whether morphological alterations are adaptive or destructive (Tkacheva et al., 2004). In addition, according to Camargo and Martinez (2007), Ayandiran et al. (2009) and Vigário and Sabóia-Morais (2014) on one hand the toxicants could induce gill histological alterations such as epithelium degeneration and necrosis. On the other hand, fish are able to develop numerous defense mechanisms, which could prevent the toxicant negative effects. These mechanisms are expressed in different morphological changes including edema, proliferation of epithelium and fusion.

The presence of edema along with the detachment of the lamellar epithelium is the first sign of pathology in fish and one of the more frequent lesions observed in gill epithelium of fish exposed to different xenobiotics (Mallatt, 1985; Thophon et al., 2003). Many studies revealed that interstitial edema is one of the more frequent lesions observed in gill epithelium of fish exposed to pollutants such as heavy metals (Karlsson-Norggren et al., 1986; Reid and McDonald, 1988; Hwang and Tsai, 1993; Sola et al., 1995; Bury et al., 1998; Karan et al., 1998; Cengiz and Unlu, 2002; Pane et al., 2004; Cengiz, 2006; Nero et al., 2006; Velmurugan et al. 2009). This histological alteration can also be due to the exposition to other kinds of pollutants, such as endosulfan (Nowak 1992), paraquat (Banaee, 2013) and drugs (Schwaiger et al., 2004). Furthermore, Karan et al. (1998); Arellano et al. (1999); Cengiz and Unlu (2002); De Boeck et al. (2001); Pane et al. (2004); Schwaiger et al. (2004) and Olurin et al. (2006) claim that lifting of lamellar epithelium is another histological change observed, probably induced by the incidence of severe edema. Laurèn and McDonald (1985); Haaparanta et al. (1997); Arellano et al. (1999); Alexopoulos et al. (2003) and Van Heerden et al. (2004) suppose that edema with lifting of lamellar epithelium could serve

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as a mechanism of defense, because separation of the epithelial lamellae increases the distance across, which waterborne pollutants must diffuse to reach the bloodstream.

Hyperplasia in the gill epithelium in different fish species induced by trace metals was reported by Arellano (1999); De Boeck et al. (2001), Van Heerden et al. (2004); Figueiredo-Fernandes et al. (2007); Velmurugan et al. (2007), Mohamed (2009) and Georgieva et al. (2014). These results were also found in fish exposed to other pollutants such as pesticides (Neskovic et al., 1993; Van den Heuvel et al., 2000; Rosety-Rodríguez et al., 2002; Oropeca et al., 2005). De Boeck et al. (2007) think that the proliferative changes can increase the water blood distance and reduce the absorption of xenobiotics, but in turn, decrease the respiratory surface area, which reduces the effectiveness of gas exchange ion uptake. According to Figueiredo-Fernandes et al. (2007) cell proliferation with thickening of gill filament epithelium is a histological change, which may lead to the lamellar fusion. Cell proliferation with thickening of the gill filament epithelium after heavy metal exposure is described by several authors (Arellano, 1999; De Boeck et al., 2001; Van Heerden et al., 2004), but these results also were found in fish exposed to other pollutants (Randi et al., 1996; Van den Heuvel et al., 2000; Rosety-Rodríguez et al., 2002). On one hand, by adapting to apparently pathological symptoms, the fish may be able to survive the pollution effects (Evans et al., 2005). On the other hand, as stated by Georgieva et al. (2014) the increase in the number of cells would cause an intense mucous secretion and thicken the mucous layer. Thus, the emergence of fusion and additional significant thickening of the filamentous epithelium could have impact on the respiration and osmoregulation processes in the fish body. Lamellar fusion was found in different fish such as Nile tilapia, *Oreochromis niloticus* exposed to treated sewage water for 96 h (Fontaínhas-Fernandes et al., 2008), in spotted snake head, *Channa punctatus* exposed to sublethal herbicide concentrations for 96 h (Butchiram et al., 2009) and in common carp, *Cyprinus carpio* exposed to 0.029 and 0.041 mg/L deltamethrin for short-term 96 h (Cengiz, 2006).

Peuranen et al. (1994) state that observations such as rupture of the branchial epithelium are considered as direct, dose-dependent deleterious effects of the pollutant, while hyperplasia, lamellar fusion and mucous hypersecretion could be signs of branchial defense responses. Epithelial rupture could lead to a negative ion balance and changes in the hematocrit and hemoglobin and could cause severe disturbances in gill respiration. This ruptur explains the ion loss from the plasma, as described by Peuranen et al. (1994). Dutta et al. (1996) observed such changes in catfish *Heteropneusteus fossilis* exposed to the 4 mg/L malathion for 24, 48, 72 and 96 h. Epithelial hyperplasia, curling and fusion of the secondary lamellae were noticed in mrigal carp, *Cirrhinus mrigala* after exposure to sublethal monocrotophos concentrations (Velmurugan et al., 2007) and in mosquitofish, *Gambusia affinis* after 30 days of exposure to 0.25–0.50 μg/L deltamethrin (Cengiz and Unlu 2006).

Thophon et al. (2003) and Garcia-Santos et al. (2006) refer that lamellar axis vasodilatation can induce changes in the normal structure of pillar cells with consequent loss of their support function and probably is responsible for the emergence of lamellar aneurysms in fish exposed to heavy metals such as cadmium. Similar results are observed by Figueiredo-Fernandes et al. (2006) in Nile tilapia, *Oreochromis niloticus*, after 0.5, 1.0 and 2.5 mg/L copper exposure for 21 days. However, Mallat (1985) suggests that these lesions are rarely associated to metals exposition. Severe vasodilatation was reported by Georgieva et al. (2014), but in common carp, *Cyprinus*

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carpio exposed to sublethal pesticides concentrations. Aneurisms are the most severe alteration in the fish venous system and they are a result from extended vasodilatation with the collapse of pillar cells and the breakdown of vascular integrity (Martinez et al., 2004; Cengiz, 2006). Garcia-Santos et al. (2006) and Stentiford et al. (2003) observed an increased frequency of this serious alteration in fish from contaminated areas and affirmed that it can be associated with the presence of toxicants such as metals in the water. Monteiro et al. (2005) proved that aneurisms might be used as a sensitive and reliable biomarker of acute copper exposure.

Degenerative changes and necrosis in the fish gill epithelium were reported in the work of Santhakumar et al. (2001), Butchiram et al. (2009) and Hasan et al. (2014) after heavy metal and pesticide exposure.

Overall, as stated by Au (2004) the response of the fish gill to inorganic and organic pollutant exposure seems not to be affected by the biology of the fish (sex and age) or seasonal factors. However, we can add that some fish species are more sensitive than others to pollutants (trout, salmon) and other are more resilient to contaminated water (carp, perch). It is also important to note that even though all of the above histological alterations can be found in fish exposed to toxicants, some of them induce more severe tissue changes than others. In general, the gill histopathology appears to be a promising biomarker for general environmental contamination, although tissue preparation for gill histopathological study is time consuming (Oliveira Ribeiro et al., 2006).

Histological alterations in fish liver

Once the toxicants cross the biological barriers and enter the bloodstream, they will reach and accumulate in the fish internal organs. Numerous studies have quantified contaminants in different fish organs to evaluate environmental quality, seeking causal relationships with fish health, and, based on these, the liver is likely to be the best choice, followed by the kidney and gills (Begun et al., 2004; Pokorska et al., 2012; Majnoni et al., 2013). According to Hinton and Laurén (1990), Van der Oost et al. (2003) and Salamat and Zarie (2012) the liver is a detoxification organ and it is essential for both, the metabolism and the excretion of toxic substances in the fish body. According to Mohamed (2009) the liver is also a target organ due to its large blood supply, which causes noticeable toxicant exposure. Moreover, the liver is reported to be the primary organ for bioaccumulation and thus, has been extensively studied in regards to the toxic effects of different xenobiotics (Hinton and Laurén, 1990; Yilmaz et al., 2007; van Dyk et al., 2007; Simonato et al., 2008; Madureira et al., 2012; Nunes et al., 2015).

According to Vicenti et al. (2005) and Figueiredo-Fernandes et al. (2007) healthy teleost fish generally exhibits a normal architecture with a typical parenchymatous appearance with no pathological abnormalities. The parenchyma itself is primarily composed of epithelial cells (hepatocytes) typically with a large central nucleus and homogenous cytoplasm. The hepatocytes are located among blood capillaries called sinusoids forming a cord-like structure known as hepatic cell cords. The lumen of sinusoids contain mainly erythrocytes. The venous blood enteres the liver caudally from the intestine via the hepatic portal veins and branches into the sinusoids. They are lined with reticuloendothelial cells, which are in turn surrounded by hepatocytes.

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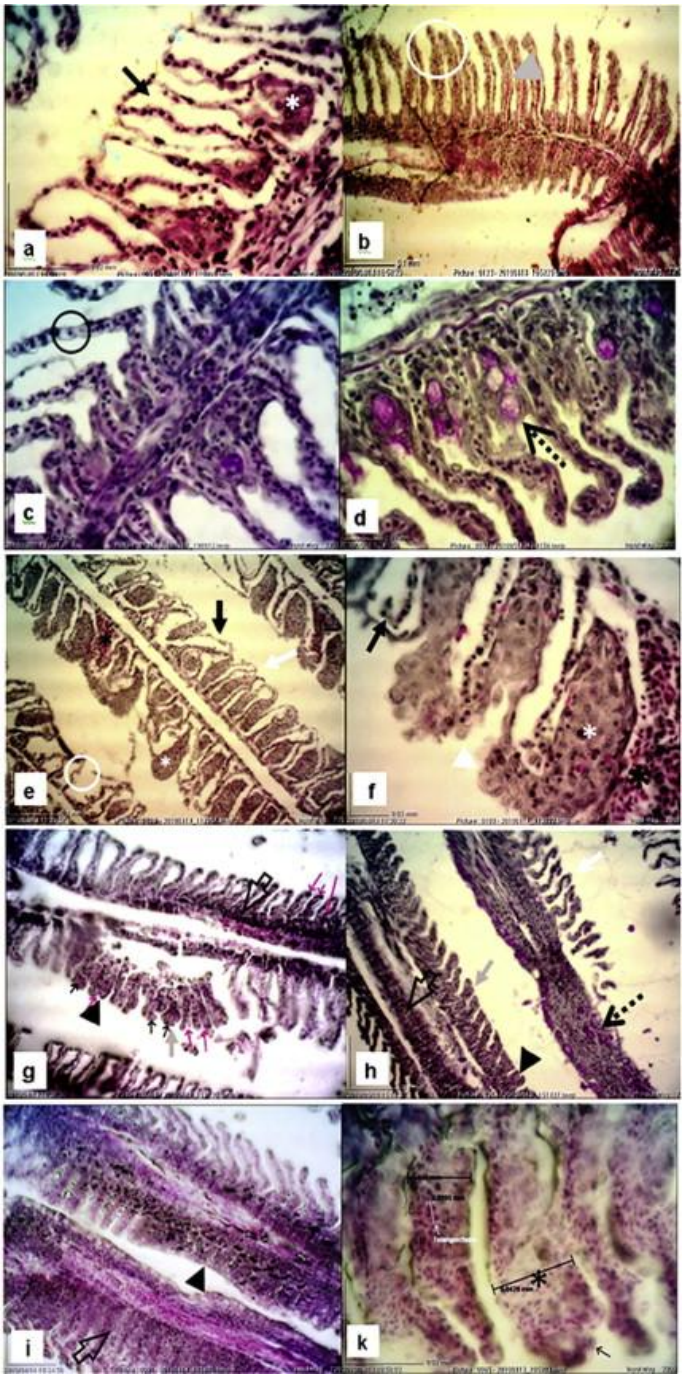

Figure 1. *Photomicrographs of histopathological alternations of gills within the A. latus groups exposed to $HgCl_2$; extensive epithelial lifting and edema of the lamellae with enlarged sub-epithelial spaces (black arrows), hyperplasia of the epithelial cells (white *) with partial fusion of the lamellae (black arrowhead), club shaping of gill lamellae (gray arrow), lamellae with the marginal channel dilated (black marked circularly), blood congestion (gray arrowhead), lamellar aneurysm (black *) within the lamellae, lamellar disorganization (white arrow), hypertrophy of the lamellar epithelium (white arrowhead), leukocytes infiltration (hollow arrow), increase of mucosal cells (dashed arrow), epithelium rupture (white marked circularly); (a–c) Sea breams treated with 10 µg/L $HgCl_2$ (group 1); (d–f) Sea breams treated with 20 µg/L $HgCl_2$ (group 2),; (g and h) Sea breams treated with 35 µg/L $HgCl_2$ (group 3); and (j and k) Sea breams treated with 50 µg/L $HgCl_2$ (group 4). (a–c, f and k) (H&E; ×2900); (e, g and i) (H&E; ×725); (d and h) (PAS; ×725) (Hassaninezhad et al., 2014).*

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Evaluation of the fish liver histopathology is a monitoring tool that can provide an assessment of the effects of environmental stressors on fish populations (Hinton and Lauren, 1990; Fernandes et al., 2008). Also, it was proposed to be one of the most reliable indicators for health impairment of aquatic animals by anthropogenic activities (Hinton and Laurén, 1990; Hinton et al., 2001; Stentiford et al., 2003), since the liver plays an important role in many vital functions (Moon et al., 1985; Triebskorn et al., 2002; Figueiredo-Fernandes et al., 2006; Lang et al., 2006). Several studies carried out in coastal waters have shown correlation between environmental contaminants, bad water quality and occurrence of toxicopathic liver lesions in fish (Vethaak et al., 1992; Vethaak et al., 1996; Stentiford et al., 2003; Feist et al., 2004; Carvalho Neta et al., 2014). Thus, according to Lang (2002) and Feist et al. (2004) in recent years, the fish diseases and liver histopathological alterations have been used as indicators of pollution effects and have been implemented in monitoring programs. Stentiford et al. (2003) state that numerous categories of liver pathology are present as reliable biomarkers of toxic damage. The presence of inflammatory lesions, hepatocellular fibrillar inclusions, and preneoplastic and neoplastic lesions is higher in fish captured in polluted environments than in fish from reference sites. However, accoreding to Pinto et al. (2009) the sort of histological alterations observed depends on individual exposition time to pollutants, as well as on pollutant type and concentration. Triebskorn et al. (1997) and Schramm et al. (1998) described methods to study the liver ultrastructure using quantitative and semi-quantitative electron microscopy.

Similarly to the gill histological alterations, the hepatic alterations are also considered as non-specific biomarkers as many different toxicants can produce liver changes. Exposure to heavy metals and different organic pollutants for example may cause histological changes in the liver and a histological investigation of exposed specimens may therefore produce meaningful results (van Dyk et al., 2007; Triebeskorn et al., 2008; Marchand et al., 2009; Hued et al., 2012; Georgieva et al., 2014). Figuieredo-Fernandes et al. (2007) suggest that high metal deposition in the fish liver leads to the abnormalities in the hepatic structure and hepatocytes, which can lead to subsequent cell death.

Wild fish hepatic lesion appearance and increment can be pointed as a sign of bad water quality and have been the subject of many studies (Vethaak et al., 1992; Simpson et al., 2000; Stentiford et al., 2003). According to Pinto et al. (2009) the sort of histological alterations observed depends on individual exposition time to pollutants, as well as on pollutant type and concentration.

According to Strüssmann and Takashima (1990) morphometric parameters such as number of hepatocytes, hepatocyte area core region of hepatocytes, glycogen content and lipids into the cytoplasm are commonly used as an indicator for hepatocyte metabolic activity.

Monteiro et al. (2005) and Rajeshkumar and Munuswamy (2011) consider that the degenerative alterations such as granular, vacuolar, hydropic and fatty degeneration are from biochemical disturbances, including the inhibition or activation of enzyme activity, changes in protein synthesis, impaired ion regulation, and the depletion of energy resources.

In scientific literature it is stated that cellular swelling occurs either directly by denaturation of volume-regulating ATPases or indirectly by disruption of the cellular energy transfer processes required for ionic regulation (Hinton and Laurén, 1990). Alterations in liver hepatocytes associated with stress were studied long ago by Metelev

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et al. (1971) who reported formation of vacuoles in the hepatocytes. Today vacuolations of hepatocytes is a common response associated with exposure to many different toxicants as described by Mishra and Mohanty (2008) and Vinodhini and Narayanan (2009). Such histological changes could represent lesions at biochemical level, including inhibition of protein synthesis and energy depletion. In addition, vacuolation of hepatocytes in mrigal carp, *Cirrhinus mrigala* was found after exposure to the pesticide lambda-cyhalothrin for 10 days (Velmurugan et al., 2007), as well as in catfish, *Corydoras paleatus* after exposure to the pesticide methyl parathion (Fanta et al., 2003). Moore et al. (1997) studied hydropic vacuolation in winter flounder, *Pleuronectes americanus* from chemically contaminated habitats. According to these authors, grossly visible foci of vacuolation correspond to the most advanced stage of this lesion and is associated with neoplasias. Similarly, Stehr et al. (1998) observed and described hydropic vacuolation in the liver of three species of bottom fishes from US West Coast (*Platichthys stellatus*, *Genyonemus lineatus* and *Lepidopsetta bilineata*). These authors observed single large, hydropic-appearing vacuoles that almost completely filled each affected cell, with little cytoplasm remaining. The edges of the vacuoles were smooth, as if they were membrane-bound, and the vacuoles appeared to be empty. The nucleus was eccentrically located within the vacuole. They also observed that this lesion can appear, in mild cases, like single cells and small groups of two to three cells affected, diffusely distributed throughout the liver. On the other hand, with smaller frequency they identified a focal form of this lesion, where nearly all of the cells within a specific area or "focus" were affected. Lang et al. (2006) found hydropic vacuolation in Baltic flounder, *Platichthys flesus*, which was used as indicator for biological effects of contaminants.

Yancheva et al. (2015) found hepatocytes, which were morphologically altered and resembled typical adipose cells with flattened nucleus located on the periphery in fish from a metal-contaminated lake. Marty et al. (2003) studied demersal rockfish species from Prince William Sound, AK, USA, after the ExxonValdez oil spill in 1989 and detected some hepatic microlesions, such as pigmented macrophage aggregates and hepatic megalocytosis, fibrosis and lipid accumulation. In that study, it was confirmed that in fish with abundant hepatic lipid (lipid accumulation), the hepatocyte cytoplasm contained variable amounts of clear, round, well-demarcated vacuoles and some cells contained multiple cytoplasmic vacuoles. According to Pinto et al. (2009) this histological change could represent several biochemical lesions, such as inhibition of protein synthesis, energy depletion, disaggregation of microtubules and shifts in substance utilization. This lesion can appear under two forms: foci of variable size or disperse by whole hepatic parenchyma. Cristiane de Melo et al. (2008) found in the cytoplasm of hepatocytes of catfish, *Rhamdia quelen* exposed to 0.01 ml/L Folidol 600 a higher presence of lipid droplets in the first 48 h than in the control fish. According to Arellano et al. (1999), this lipid deposition can indicate a possible alteration in the fat metabolism of the hepatocytes. In the fish thinlip mullet, *Liza ramada*, Biagianti-Risbourg and Bastide (1995) described different sizes of lipid droplets according to the time of sub-lethal exposure to the herbicide atrazine.

Several studies demonstrated that alterations in number, size and shape of the hepatocyte nucleus can be due to contaminants. Alterations in the size of nucleus were previously regarded by Paris-Palacios et al. (2000) in zebrafish, *Brachydanio rerio* exposed to sub-lethal concentrations of copper sulphate. Braunbeck et al. (1990 a,b) state that alterations in size and shape of nucleus have often been regarding as signs of

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increased metabolic activity, but may be also of pathological origin. Ahmad et al. (2002) claim that disturbances in the osmotic regulation of cellular membranes result in increasing the volume of the nuclei and nucleoli, and this lead to necrosis of liver cells. Ayoola (2008) found that the fish liver exposed to glyphosate had infiltration of leukocytes, increased hepatocyte size with pyknotic nuclei and presence of vacuoles. Alterations such as irregular shaped hepatocytes and cytoplasmatic vacuolation were also described in populations of mullets (Coz-Rakovac et al., 2008) and sea bass (Coz-Rakovac et al., 2005) aggregated around fish farms.

Varanka et al. (2001) suggest that metal accumulation in the liver of common carp, *Cyprinus carpio* causes hepatocyte lysis, cirrhosis and ultimately death. Braunbeck (1994) reported proliferation of lysosomes in the hepatocytes of rainbow trout (*Oncorhynchus mykiss*) following in vivo and in vitro sub-lethal exposure to xenobiotics (e.g. 4-chloroaniline). Nero et al. (2006 a,b) conducted a study with yellow perch, *Perca flavescens* and caged goldfish, *Carassius auratus* held in waters containing high levels of oil sands process-affected water and observed that following a 3-week exposure, liver histopathological change were observed in the individuals. In relation to significant changes in liver histopathology, it was dominated by degenerative alterations for both species and, to a lesser extent, inflammatory (yellow perch) and cytoplasmic (goldfish) alterations. Necrotic foci and nuclear pleomorphism dominated the degenerative lesions in both fish species.

Figueiredo-Fernandes et al. (2006) and Ada et al. (2012) found increased macrophages and eosinophil cells in Nile tilapia, *Oreochromis niloticus* liver under sub-lethal paraquat exposure.

Do Carmo Langiano and Martinez (2008) found degenerative changes in the hepatic cytoplasm and nuclei, as well as hyperemia in liver of rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) after 7.5 and 10 mg/L Roundup exposure for 6, 26 and 96 h.

Ramírez-Duarte et al. (2008) determined LC_{50} for glyphosate for Amazonic pacu, *Piaractus brachypomus*. At these concentrations the authors found degenerative changes in the fish liver such as hyaline droplets and lipid vacuolation in the cytoplasm. Walter et al. (2000) and Matos et al. (2007) also determined lipid accumulation in liver of Nile tilapia, *Oreochromis niloticus* after chronic exposure with dioxin and carbaril, respectively. Similar results were also reported by Gultekin et al. (2000) and Teh et al. (2005). Gaafar et al. (2010) found single hyaline droplets, combined with vacuole degeneration and necrosis in fish liver after fungicide exposure for 96 h.

El-Serafy et al. (2009) determined reduction in the carbohydrates content in the liver of Blue tilapia, *Oreochromis aureus* after sub-lethal (20%, 40% and 80% of LC_{50}) phenol exposure for 7 days. Similar results were reported by Pathan et al.(2009 a,b), De Boeck et al. (2010) and Wiseman and Vijayan (2011) after other organic contaminants. On the other hand, Ramesh and Saravanan (2008) found increased glycogen level in the liver of common carp, *Cyprinus carpio* after chlorpyrifros treatment.

According to Simenova (1999); Rabitto et al. (2005); Mela et al. (2007) and Carrola et al. (2009) the presence of necrotic areas is one of the most serious alterations in liver structure that can occur under the influence of metals. Roberts (1989) explains that necrosis can be characterized by nuclear and cytoplasmic alterations, followed by loss of the cellular contours. When the necrosis of a cell occurs there is the release of chemical signals that induce cell proliferation for the substitution of the dead cells, and so the tissue does not loose its structural and functional condition. Manahan (1991) state

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that the occurrence of necrosis is also a consequence of enzymatic inhibition, damages in the cellular membrane integrity, and disturbances in the synthesis of proteins and carbohydrate metabolism. In fish liver, the presence of necrosis area is also related with xenobiotic concentration during the detoxifying process. Ayoola (2008) and Olufayo and Alade (2012) consider that necrosis of some areas in the liver tissue were probably resulted from the excessive work required by the fish to get rid of the toxicant during the process of detoxification by the liver. Cengiz and Unlu (2006) determined hepatic necrosis in mosquitofish, *Gambusia affinis* due to sublethal effects of deltamethrin. Necrosis focuses were also observed in the liver of the fossil cat, *Heteropneustes fossilis* (Dutta et al., 1993), zebrafish, *Danio rerio* (Rodrigues and Fanta, 1998) and rainbow trout, *Onchorynchus mykiss* (Uguz et al., 2003) exposed to different pollutants.

Devi and Mishra (2013) and Chamarthi et al. (2014) found leucocytes infiltration in *Channa punctatus* and *Cyprinus carpio* liver under chlorpirifos and kinalfos exposure. In addition, Al-Mamoori et al. (2014) determined such histophatological changes, as well as, vasodilatation in small blood vessels and necrosis in *Cyprinus carpio* liver after acute and chronic exposure with 0.05 mg/L, 0.1 mg/L, 0.25 mg/L chlorfos.

Yancheva et al. (2015) found venous hyperaemia in perch from a metal-contaminated lake, which was assumed to be linked to disturbances in the hepatic blood circulation. It was presented in the major and small blood vessels, as well as hepatic sinusoids, most likely due to common vein congestion. Van Dyk et al. (2007) consider that liver hyperemia can lead to hepatic necrosis and atrophy. McHugh et al. (2011) found that the liver alterations in fish exposed to pesticides were mostly associated with circulatory disturbances, related to pathological conditions of blood and tissue fluid flow and regressive changes. They included dilation of blood sinusoids, as well as cytoplasmic granular degeneration and vacuolation of the hepatocytes.

Overall, similarly to the histological alterations in the gills, we can conclude that there are many different (mild or severe) liver changes, which can be induced by both, organic and inorganic toxicants. It also seems that most of them are destructive rather than adaptive. This can be linked with the inability of the detoxification organ to get rid of the contaminants, thus also indicating biochemical disturbances in the fish body.

Histological alterations in fish kidney

The vertebrate kidney is the main organ involved in the maintenance of body fluid homeostasis. The morphology and function of the kidney have been modified through evolution to fulfill different physiological requirement and the widest range of kidney types is found in fishes (Hentschel and Elger, 1989).

In teleosts, the kidney, together with the gills and intestine, are responsible for excretion and the maintenance of the homeostasis of the body fluids (Hinton et al., 1992; Evans, 1993; Ojeda et al., 2003) and, besides producing urine, acts as an excretory route for the metabolites of a variety of xenobiotics to, which the fish may be exposed (WHO, 1991; Hinton et al., 1992; Eisler, 1998). The kidney also excretes other nitrogen-containing waste products from the metabolism such as ammonia and creatinine. In addition, in fish as in higher vertebrates, the kidney performs an important function related to electrolyte and water balance and the maintenance of a stable internal environment (Cengiz, 2006).

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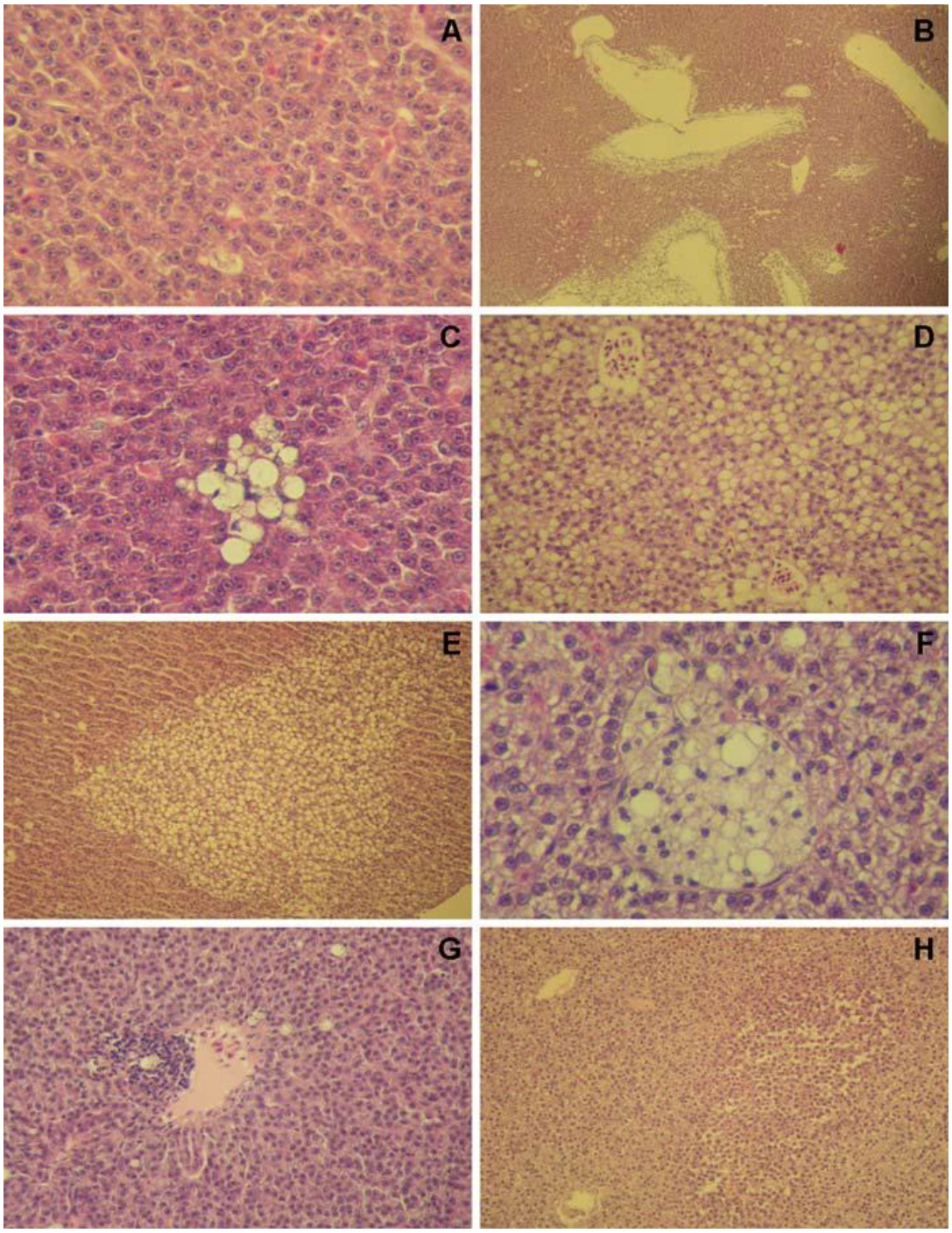

Figure 2. *Liver light microscopy of histological sections of gudgeon (G. gobio) caught in Febros River. A Hepatic parenchyma (reference), where it is shown the homogeneity of hepatocytes distribution (H&E-×800); B relatively large perivascular necrosis (H&E-×80); C hydropic vacuolation foci, showing a small aggregate of large vacuoles, with hydropic appearance and where the hepatocyte nuclei have an outlying position (H&E-×800); D lipidic vacuolation diffusely distributed in the hepatic parenchyma (H&E-×400); E lipidic vacuolation foci, aggregates of several small, clear, and rounded vacuoles (H&E-×200); F unspecific granuloma, where it is possible to observe the clear and homogeneous acelullar content, with a well defined margin (H&E-×800); G small lymphocytic foci close to a blood vessel (H&E-×400); H disaggregated cells foci, in this area, the hepatocytes are slightly moved away from each other and their walls seems to be disintegrated (H&E-×400) (Pinto et al., 2009).*

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Mobjerg et al. (2004) describe that the kidney of all vertebrates is made up of nephrons, which are the functional, structural and adaptation process. Teleostean kidney consists of anterior kidney (also known as head kidney) and posterior kidney (also known as body or trunk kidney). Embriologically, anterior kidney derives from pronephros, and posterior kidney from mesonephros (Takashima and Hibiya, 1995).

The anterior kidney is integrated into the endocrine system of the fish and is very important for the stress response, mediated by the hypothalamus–pituitary–interrenal cell (HPI) axis and by the hypothalamus–sympathetic nervous system–chromaffin tissue (HSC) axis (Donaldson, 1981; Wendelaar Bonga, 1997).

In addition, the head of kidney contains endocrine elements - the chromaffin cells and interrenal tissue, which are located around the blood vessels. The posterior kidney contains the nephrons with variable quantities of hemopoietic and lymphoid tissue in the interstitium. It is mainly composed of renal corpuscles that are made up of the Bowman's capsules, glomeruli, renal tubules and collecting ducts. In vertebrates, three types of kidney are found: the pronephros, mesonephros and metanephros (Federova, 1998; Mobjerg et al. 2004).

The morphology of the kidney of each species of teleost often reflects the osmotic demands placed on the organism by the environment (Hickman and Trump, 1969). Thus, the freshwater fishes are faced with the need to produce copious amounts of dilute urine and, therefore, their kidneys have well developed renal corpuscles and both, proximal and distal segments in the tubules (Hentschel and Elger, 1987). In contrast, marine fishes have to conserve water and produce small amounts of concentrated urine and consequently their kidneys are either aglomerular (no renal corpuscles) or with poorly developed renal corpuscles (Yousin et al., 1989).

The kidney in fish receives the largest proportion of postbranchial blood, and therefore renal lesions might be expected to be good indicators of environmental pollution (Hinton and Laurén, 1990; Ortiz et al., 2003; Kurtovic et al., 2008). Therefore, Hinton et al. (1992); Oliveira Ribeiro et al. (1996); Schwaiger (2001) and Pacheco and Santos (2002) consider that the effects of pollutants on fish kidneys have been studied in some species and the severity of damage seen depends on the sensitivity of the species to the substances released into the environment (similarly to the gills and liver).

Most common alterations found in the kidney of fishes exposed to water contamination are tubule degeneration (cloudy swelling and hyaline droplets) and changes in the corpuscle, such as dilation of capillaries in the glomerulus and reduction of Bowman's space (Takashima and Hibiya, 1995). Following exposure of fish to toxic agents such pesticides, histological alterations in the fish kidney were found at the level of the tubular epithelium and glomerulus (Teh et al., 1997; Thophon et al., 2003). Yildirim et al. (2006), mentioned that hyaline droplets formation results from tubular reabsorption of plasma protein lost to the urine by glomerular damage. Hyaline degeneration in kidney was observed by Boran et al. (2010) in rainbow trout *Oncorhynchus mykiss*, after 0.10 mg/L to 2.00 mg/L maneb and 0.20 mg/L to 3.90 mg/L carbaryl exposure for 24, 48, 72 and 96 h.

Shrinkage of the glomerulus and increased space within the Bowman's capsule were also observed by Ortiz et al. (2003) after lindane exposure. Distal convoluted tubules decreased in size and formations of vacuoles were reported by Tilak et al. (2005) after butachlor exposure for 10 days.

Exposure to metals frequently causes alterations in the tubules and glomerulus, such as was described by Thophon et al. (2003) in perch, *Lates calcarifer* exposed to sub-

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lethal cadmium. Handy and Penrice (1993) found swollen Bowman's capsule cells and melanomacrophages in the kidney of trout, *Salmo trutta* and tilapia, *Oreochromis mossambicus* exposed to mercuric chloride. Similar alterations were found in fishes exposed to organic contaminants (Veiga et al., 2002) and mixed environmental contaminants (Schwaiger et al., 1997; Pacheco and Santos, 2002).

Dilation of the lumina of the kidney tubules, necrosis of tubules, shrinkage of glomerular tuft, vacuolation blood cells in the glomerular tuft have been reported in fossil cat, *Heteropneustes fossilis* exposed to chlorpyrifos for 96 h (Srivastava et al., 1990). Elsan treatment in *Channa punctatus* resulted in a significant decrease in the dimension of Bowman's capsule and glomerulus, and the tubules lost their regular shape due to precipitation of cytoplasm and karyolysis for 90 days (Banerjee and Bhattacharya, 1994). Dass and Mukherjee (2000) reported dilation of tubules, necrotic changes characterized by karyorrhexis and karyolysis at the nuclei of affected cells of carp, *Labeo rohita*, exposed to 1/10 and 1/5 sub-lethal doses hexachlorocyclohexane after 45-day test period. Ortiz et al. (2003) showed tubular necrosis, desquamation and vacuolisation of tubular epithelial cells in *Mugil sp*., *Cyprinus carpio* and *Barbus sp*. kidney exposed to lindane (γ-Hexachloro-cyclohexane – γ-HCH-).

Lauren et al. (1989) found tubular degeneration and eosinophilic, proteinacious, intratubular casts and hyaline droplets, and an increase in the amount of hemosiderin or melanin-like intertubular deposits in rainbow trout, *Salmo gairgneri* fed with the antibiotic fumagilin. Hyaline droplet formation results from tubular reabsorption of plasma protein lost to the urine by glomerular damage. Intratubular casts are markers of damage to the tubule cells themselves. Intramuscular injection of gentamycin sulphate resulted in thickening and sloughing of the glomerular epithelium in Channel catfish, *Ictalurus punctatus* (Rolf et al., 1986).

Vesilek et al. (2010) found that the caudal kidney of carp exposed to sub-lethal terbutryn showed cellular alterations such as destruction of the tubules in the caudal kidney. Fischer-Scherl et al. (1991) observed changes in renal corpuscules and tubules of rainbow trout after chronic exposure to atrazine.

McHugh et al. (2011) observed dilation of the glomerulus capillaries, vacuolation of the renal tubule and hyaline droplet degeneration in kidney of tigerfish, *Hydrocynus vittatus* from a DDT-affected area.

Schmidt-Posthaus et al. (2001) observed in brown trout exposed to polluted river water a higher prevalence and severity of tubulonephrosis, deposits of desquamating tubular cells, hyalinous casts in the tubules and peritubular fibroblast proliferation.

Camargo and Martinez (2007) found glomerular expansion and absence of the Bowman's space, and tubule cells with hypertrophied nucleus, tubule starting the regeneration process, occlusion of the tubular lumen and cloudy swelling degeneration in a Neotropical fish caged in an urban stream.

In their study Ptashynski and Klaverkamp (2002) observed histological alterations in the posterior kidney of white fish fed with high dose diets, indicating that kidney may be a target organ for nickel toxicity. In the control fish, the trunk kidney involved in excretory functions was formed by a large number of nephrons each having a renal corpuscle. The corpuscle was the proximal part of the tubule, which consists of two parts, a glomerulus and a capsule. The histopathological abnormalities in kidney of silver carp, *Hypophthalmichthys molitrix* were time dependent. The kidney of fish treated with nickel for 10 days, exhibited tubules with hyperplasia and hypertrophic nuclei and haemolysis of erythrocytes. In fish after 20 days of exposure the tubules were

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seen with cytoplasmolysis, karyolysis and vacuolization. At the end of 30 days treatment, ruptured cells, syncytical condition and pyknotic nuclei with aggregation of nuclei were seen due to the damage of plasma membrane. The glomerulus structure was disruptured, and the convoluted and uriniferous tubules are enlarged.

Athikesavan et al. (2006) also observed hypertrophy, hyperplasla, haemolysis, vacuolization, karyolysis, ruptured cell and pyknotic nuclei in the kidney of silver carp, *Hypophthalmichthys molitrix* exposed to sublethal concentrations of nickel after 30 days.

According to Weber et al. (2003) the occurrence of dilated tubules appears to be a consequence of dead and dying epithelial cells while a thickening of Bowman's capsule can arise as a result of fibrosis.

Pal et al. (2011) observed dilation of the glomerular capillaries and hemorrhage in the Bowman's space were noted in the renal corpuscles of exposure groups, necrosis of the tubular epithelium cells in the fish kidney exposed to dietary heavy oil. Similar structural changes were observed in brown trout, *Salmo trutta f. fario* and stone loach, *Barbatula barbatula* (Schwaiger et al., 1997) and in *Astyanax altiparanae* (Silva and Martinez, 2007) collected from polluted urban stream containing high amount of PAHs and other chemicals.

Glomerular lesions, shrinking of the glomerulus and enlargement of space inside Bowman's capsule, dwindling of the tubular lumen, degeneration and necrosis of renal tubules were observed in the kidney tissue of rainbow trout, *Oncorhynchus mykiss* after the exposure of 0.1 and 0.2 mg/L diazinon for 28 days period by Banaee et al. (2013). Gabriel et al. (2007) reported cellular hypertrophy, tubular degeneration, enlarged glomerulus and tubular necrosis in African catfish, *Clarias gariepinus* with a dose-dependent relationship to fuel oil.

Silva and Martinez (2007) found that the histological alterations in the posterior kidney of *Astyanax altiparanae* from the investigated sites were in complete contrast to those from the reference site, in respect of the type, severity and number of lesions observed. Tissue changes in the reference fish were light, whereas the lesions found in fish from the studied stream were more severe and in some cases irreparable, such as necrosis of the tubule epithelial cells, reflecting the poor water quality of this urban stream.

In general, the morphological structure of fish kidneys is well-studied. However, on the basis of the observed literature we can say that in the histopathological studies, the kidneys are not as widely used as the gills and liver but yet the results are very useful in terms of water contamination and its effects on fish.

Conclusions

Overall, we can conclude that the histopathological alterations in target fish organs such as the gills, liver and kidneys have been successfully applied in ecotoxicological research and risk assessment programs in the last few decades. Even though, these biomarkers are non-specific, time-consuming (but relatively cheap) and require well-trained and knowledgeable experts, they provide accurate data on the effects of different contaminants which on the other hand, can signal for alterations at lower biological organization. We therefore, suggest that histopathology should be more often included in monitoring programs on contaminated aquatic systems, along with other biomarkers and chemical analyses of waters and sediments.

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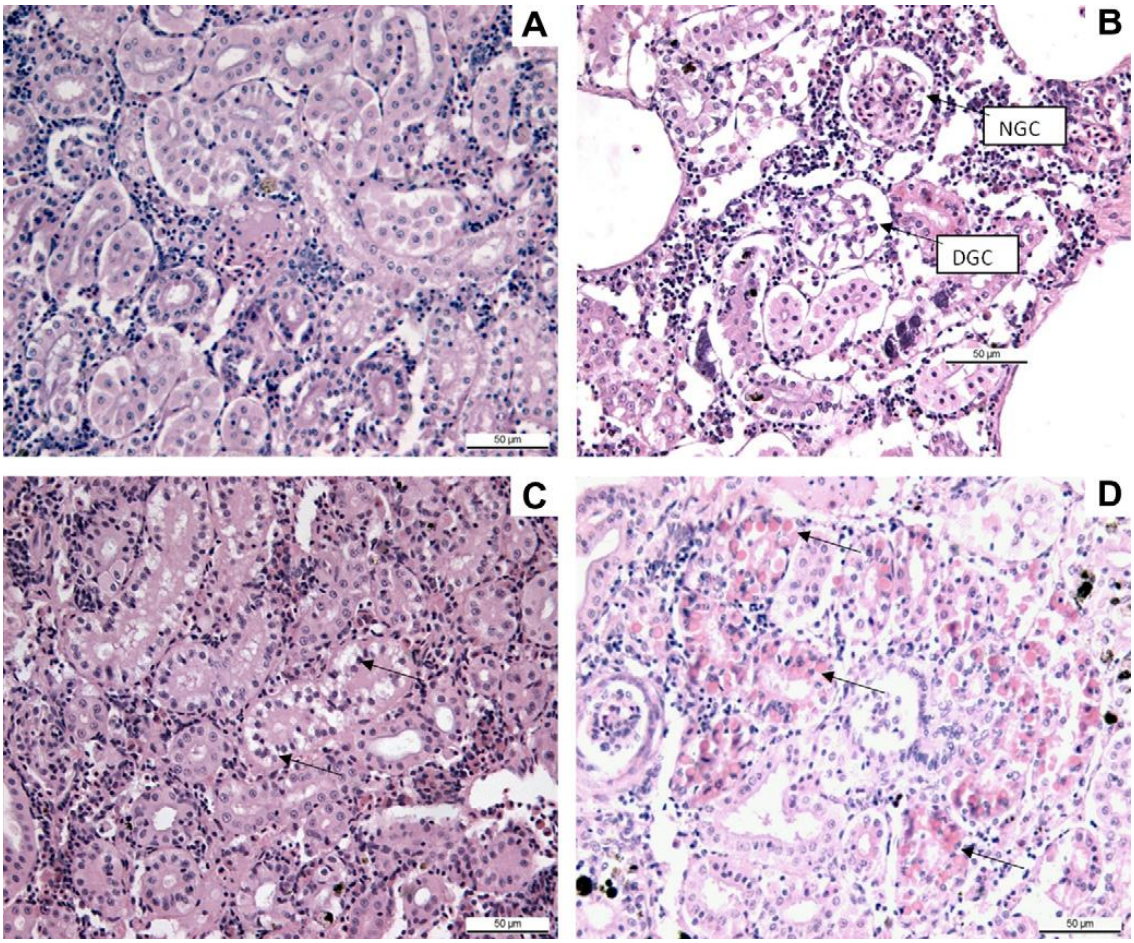

Figure 3. *Micrographs of H. vittatus kidney sections (5 µm) stained with H&E: (A) Normal kidney; (B) dilation of the glomerulus capillaries. Normal glomerulus represented by NGC and dilate glomerulus represented by DGC; (C) vacuolation of the renal tubule (arrows); (D) hyaline droplet degeneration (arrows)(McHugh et al., 2011).*

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