www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 16: 241-250 (2016) DOI: 10.4194/1303-2712-v16_2_03

RESEARCH PAPER

Liver Pathology of Female Ohrid Trout (*Salmo letnica* Kar.) from the Eastern Coast of Lake Ohrid: Baseline Data Suggesting the Presence of a Pollution Gradient

Maja Jordanova[1],*, Katerina Rebok[1], Eduardo Rocha[2]

[1] Ss. Cyril & Methodius University, Faculty of Natural Sciences and Mathematics, Institute of Biology, Laboratory of Histology and Embryology, Arhimedova 3, 1000 Skopje, Republic of Macedonia.
[2] University of Porto, Institute of Biomedical Sciences Abel Salazar, Laboratory of Histology and Embryology, ICBAS, U.Porto, 4050-313 Porto, Portugal.

* Corresponding Author: Tel.: +389.23 2496 37; Fax: + 389.23 228141;
E-mail: majaj@pmf.ukim.mk

Received 08 January 2016
Accepted 14 March 2016

Abstract

Ohrid trout stock levels have been declining for decades in the Europe's oldest lake, Lake Ohrid, between Albania and Macedonia. Limited datasets indicate that this decrease is linked to overfishing and pollution. Histopathological hepatic lesions in fish have been regularly strongly associated with exposure to chemical contaminants. We collected baseline information on the toxicopathological and other lesions on this species liver, from 3 sites adjacent to 2 highly urban and 1 much less urban areas along the lake's eastern coast during 2000–2002. In addition, changes in the relative volume of pigmented macrophages were used as proxies of immune-mediated responses. The percentage of fish with pathological changes declined steeply from the 2 urban sites (40% and 27%) to the pristine site (7%). In addition, fish with lesions tended to have greater amounts of pigmented macrophages, with fish from the pristine site having significantly fewer lesions. While the number of fish analyzed was limited (n = 70), the biomarkers suggest the presence of a pollution gradient (decreasing towards south) along the eastern coast of Lake Ohrid. Despite scarce, other kind of data agree with our hypothesis. Variations of pigmented macrophages and liver toxicopathic injuries warn of the health impacts of urbanization on this endangered trout. This study supports the need to initiate a long-term biomonitoring program for this species as an index of the health status of Lake Ohrid.

Keywords: Ohrid trout, pathology, pollution, macrophages, stereology.

Introduction

Lake Ohrid was declared a UNESCO World Cultural and Natural Heritage Site in 1980, with conservation efforts being supported by the World Bank (The World Bank Group 2005). The oldest lake in Europe has a total surface area of 358.2 km^2 and is shared by the Republics of Albania and Macedonia (Spirkovski *et al.*, 2000). The lake has high ecological and evolutionary value, because it contains unique life forms dating to the last Ice Age. This natural heritage site is regularly termed a "museum of living fossils," which is attributed to its geographical isolation, and the more or less stable environmental conditions. However, the environmental conditions are not static, with humans playing a major role in causing environmentally deleterious changes. Such changes include the continuous loading of water with chemical xenobiotics released by both urban communities and industry. Local (often called "fossil") Ohrid trout, *Salmo letnica,* stocks have been consistently decreasing over the last decades, despite annual repopulation efforts from artificially spawned juveniles. Lack of published data on wild stocks and fisheries is appalling, but a recent report supports that declines continue both in Albania and in Macedonia (Prifti and Cake 2013). Heavy fishing pressure and water pollution are viewed as probable causes of the decline in this species; however, existing quantitative data are limited. Pollution levels in the lake have been rated from moderate to critical in some locations, and are based on measurements of phosphorus overload, bacterial indexes, and other biological indexes (Roganovic-Zafirova *et al.*, 2001/2002; Grupce 2004; Vasilevska, Novevska, and Naumovski 2004). Ongoing eutrophication is viewed as one major threat to the Lake Ohrid (Matzinger *et al.*, 2006; Schneider *et al.*, 2014).

Previous research has shown the presence of histopathological changes in several wild fish species (other than Ohrid trout) collected from Lake Ohrid (Roganovic-Zafirova and Jordanova 1998/99, 1999; Roganovic-Zafirova and Tavcioska-Vasileva 1998/99; Roganovic-Zafirova *et al.*, 2001/02, 2003). The authors of these studies suggested that the lesions were the result of an increase in the release of toxicants in the water via various anthropogenic activities. However, limited research has focused on determining whether geographic patterns of pollution reflect those of affected fish. Here, we focused on the pollution-related pathology screening of Ohrid trout, which represent the most important economic species in the lake (until 2004/2005, as all sort of captures have been forbidden since then in Macedonia), and one of its most ecologically important species. It feeds on smaller teleosts, invertebrates (viz. chironomid larvae), zooplankton, and even its own eggs (Stefanovic 1948; Naumovski 1995). As a top predator fish, the salmonid are more prone to pollutant bioaccumulation, resulting in associated biological effects (Savini *et al.*, 2010; Zananski *et al.*, 2011). Data are very rare or not available on the types/levels of pollutants close to the urban centers, the major sources of contaminants, nor the "cleanest" areas of Lake Ohrid. Hence, we collected trout from locations that were expected to be subject to different levels of pollution (i.e., adjacent to urban and touristic sites versus poorly populated sites backed by natural vegetation). The trout were collected through field-work conducted between 2000 and 2002, which mainly focused on other objectives, viz. associated with the female breeding cycle (Jordanova 2004; Jordanova *et al.*, 2007, 2008, 2009); and that is why trout males were not captured at the time (thus impairing inter-gender comparisons herein). Anyway, we hypothesized that if lake pollution levels were fairly uniform, fish from different locations should have similar frequencies/types of hepatic lesions. As environmental contaminants often induce cell mediated responses, such as proliferation of macrophages (MACs), and that gains in pigmented MACs can be sensible general indicators of fish living in degraded habitats (Blazer *et al.*, 1994; Fournie *et al.*, 2001; Agius and Roberts 2003), we also hypothesized that the magnitude of pigmented MACs in the liver would be associated with lesions and/or the location of fish collection.

Material and Methods

Ohrid trout (*Salmo letnica*; n = 70, range 480–1340 g) adult females were obtained from legal fisherman at Lake Ohrid (41°05′N, 20°45′E), from June 2000 to September 2002. The fish were captured at 3 sites (Figure 1): (1) Andon Dukov-Virce, which is located near an urban community with zones of multiple contamination inputs, mainly from the touristic town of Ohrid — with 200,000 permanent residents plus tens of thousands tourists coming in summer, and with the nearby coast rated as very polluted because of the very high *Escherichia coli* counts (LOCP, 2002); (2) Pestani, which is located in a highly touristic embayment with suspected waste water disposal based on public knowledge and media coverage, although supporting international scientific publications are not available — classified as polluted in view of the high load of *Escherichia coli* (LOCP, 2002); and (3) Veli Dab-Trpejca, which is located in a much less developed embayment, was used as the reference site for this study. The distance between Andon Dukov-Virce and Pestani coastal areas is about

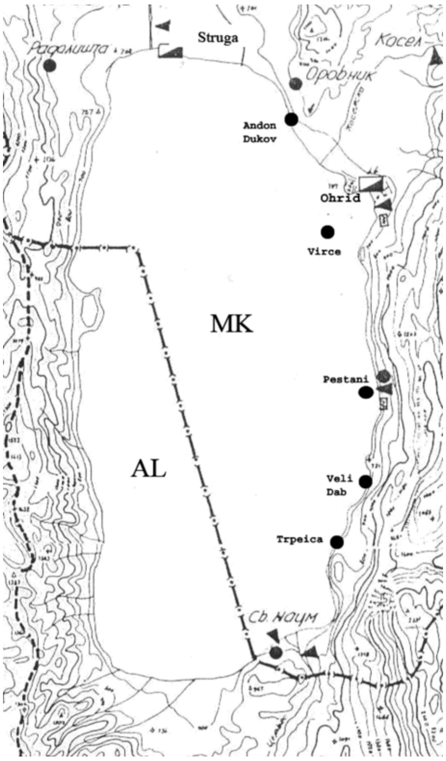

Figure 1. Map of the Ohrid Lake, showing the geographic distribution of the sampling sites. MK and AL indicate the Macedonian and Albanian parts of the Lake, respectively.

13 km, while the distance between Pestani and Veli Dab-Trpeica coastal areas is approximately 8 km. According to fish accessibility, a total of 14–41 individuals were sacrificed per site. Each animal was rapidly killed after capture by severing the spinal cord, just behind the opercula, and then the total length (TL), fork length (FL), and body weight (BW) were measured. The Fulton's condition factor (CF) was later calculated according to the following formula: $CF = 100 \times BW\ (g) \div FL^3\ (cm)$ (Nash, Valencia, and Geffen 2006). After the fish were measured, a visual assessment was made to identify the presence of any external gross lesions. The fish were then dissected and macroscopically inspected for any abnormalities of the visceral organs. The single-lobed liver was removed and chopped in 3- to 4-mm-thick pieces. At least 6 randomly selected pieces from each organ were collected per animal. For the smallest fish (<300 g), the entire liver was sampled. For generating a representative sample of the liver as a whole, the fragments were taken from the anterior to the posterior parts of the liver, and from central and lateral portions too. The samples were immersed in Bouin's fixative for 48 h. The samples were then routinely processed for embedding in paraffin, cut into 5-µm sections, stained with hematoxylin and eosin (H&E), and finally observed under a light microscope. The histological slides were coded so that the researcher making the diagnosis (Maja Jordanova, 2015) did not know either the location or the specific fish that was being analyzed; general diagnosis adopted published criteria (Noga 2010; Klatt and Kumar 2010; Roberts 2012).

Evaluation of the relative volume of pigmented MACs was made by stereology using differential manual point counting (Freere and Weibel 1967). From each block, sections were randomly selected, and observed at a final magnification of ×400. Twenty systematically sampled fields per section were then quantified. The counting process was made directly on the microscope, using an ocular eyepiece graticule showing a square lattice grid with 180 points. The relative MAC volume (expressed as a percentage) was estimated using the following formula:

V_V (structure, reference space) — $V_V\ (s, r) = [P(s) \times 100] \div P(r)$,

where V_V (s, r) is the percentage of the total volume of a reference space occupied by one particular given structure within that space, P(r) is the number of points that occur over a chosen structural component (in this case, MACs), and P(s) is the total number of test points hitting above the reference space (in this case, the whole liver). One hundred fields per fish were studied.

Statistical analyses were made using the software Statistica 7.0 for Windows. Differences were considered significant when $P<0.05$. To decide whether there was a significant difference in the percentage of affected fish (i.e., lesion sample prevalence) among the 3 sites, we used the Student's T-test for proportions and the Z-test for proportions (that offered equal inferences as to statistical significances). To study the effect of fish origin (i.e., sampling location) on the relative volume of MACs, we used a one-way ANOVA, followed by the post hoc Bonferroni test. To verify whether there was a significant difference between affected and non-affected fish in a particular site or in all sites pooled, we used the Student's T-test for independent samples. We did not use two-way ANOVA with all sites as in one of them we found only one fish with lesions (see Results). Yet, we did a parallel two-way ANOVA with those sites that had affected and non-affected fish, and using the cited post hoc test. Because the latter produced equal results as the Student's T-Test, regarding differences between affected and non-affected fish, for the sake of simplification we mention in the Tables only the Student's T-Test. Normality and homogeneity of variances were checked by the Shapiro-Wilk and by the Levene tests, respectively; logarithm transformation of data was made when needed, to grant the assumptions of the parametric tests.

Results

The morphometric data of the trout at each location and liver lesion status are presented in Table 1. Fish BW did not differ significantly among

Table 1. Ohrid trout morphometry: body weight (BW), total length (TL), and condition factor (CF) at the 3 study sites in Lake Ohrid[1]

Geographic Areas (sites)	BW (g)		TL (cm)		CF	
	No lesioned liver	*Lesioned liver*	*No lesioned liver*	*Lesioned liver*	*No lesioned liver*	*Lesioned liver*
Andon Dukov – Virce (n = 15)	870 (0.05)	920 (0.32)	43 (0.10)	44 (0.09)	1.4 (0.05)	1.3 (0.26)
Pestani (n = 41)	720 (0.87)	930 (0.68)	39 (0.22)^{a}	44 (0.21)	1.3 (0.21)^{A}	1.2 (0.15)^{B}
Veli Dab – Trpejca (n = 14)	1100 (0.26)	800 (n.a.)[2]	47 (0.09)^{b}	45 (n.a.)[2]	1.2 (0.01)	1.2 (n.a.)[2]
All Sites (n = 70)	840 (0.63)	910 (0.57)	42 (0.19)	44 (0.17)	1.3 (0.13)^{C}	1.2 (0.19)^{D}

[1] Values are expressed as means (coefficient of variation). [2] Data not available (n.a.) because n = 1. For every metric, different uppercase subscript letters represent differences between fish with or without liver lesions, within sampling site (read horizontally), and different lowercase subscript letters represent differences between sampling sites regarding the fish with the same lesion status (read vertically), according to ANOVA, followed by the post hoc Bonferroni test; a vs b, p = 0.004. For the All Sites, uppercase subscript letters represent differences between fish with or without liver lesions: A vs B, p = 0.020; C vs D, p = 0.038, by the Student's T-test.

locations, or with respect to liver lesion status. In contrast, TL had a significantly lower mean value at Pestani (tourist embayment) compared with Veli Dab-Trpejca (reference site). CF was significantly lower in fish with liver lesions at Pestani. Considering all sites combined, CF was similarly significantly lower in fish with hepatic injuries.

External and internal macroscopic abnormalities were not found. In contrast, lesions were found under the microscope, mostly of a non-neoplastic nature. In addition, some pre-neoplasms/ early-stage neoplasms and parasites were found. Inflammatory changes, such as lymphocyte infiltration, were often present. These changes primarily occurred around the stromal tracts (vascular and/or biliary), and tended to be associated with necrosis and fibrotic tissue around these tracts.

Hepatocellular death (at times with ballooning) was frequently observed, either in the form of necrotic areas around veins or as randomly scattered foci. The necrotic zones (with little or no inflammation) tended to be composed of unstained/poorly stained and irregularly shaped cells. Curiously, darkly stained round or irregular bodies were found at the periphery of some foci or in cell debris (Figure 2). Some of the basophilic bodies appeared to be located inside dead cells. In addition, larger basophilic bodies were cellular in nature, sometimes with a subtle spongy (tiny vacuolated) appearance; by their morphological appearance, the strange bodies were suspected to be amoebic infections (Dyková and Lom 2004). Often, biliary ducts and the nuclei of epithelial biliary cells were damaged in areas of hepatocellular death (Figure 2).

One frequently observed lesion (46% of fish) was a notorious and extensive increase in connective tissue; that was clearly distinct from the normally occurring bigger vascular-biliary tracts. That phenomenon (cholangiofibrosis) was mainly associated with stromal tracts containing biliary ducts. Thirty-one percent of fish exhibited evident accumulations of inflammatory cells (namely of lymphocytes) in the thickened stromal tracts, appearing in the form of distinct peri-cholangiolar “lymphocytic sleeves.” The morphology of cholangiofibrosis included either (1) a composite of biliary ducts within a moderately fibrous stroma or (2) large cholangiofibrotic lesions, with proliferating epithelia, at times with some luminal epithelial cells containing pycnotic nuclei. The second form of cholangiofibrosis had a more prominent fibrous capsule composed of connective tissue. The floating biliary epithelial cell debris was observed in the lumina of tracts where the amount of connective tissue had not increased. Cholangiofibrosis might be accompanied with noticeable bile duct hyperplasia, where numerous and contiguous biliary ducts, with abundant branches, were randomly distributed in the parenchyma. Some of the focal bile duct proliferations were diagnosed as early cholangiomas. Although these proliferations did not have a nodular appearance, they were composed of clusters of proliferative biliary cells forming distorted ductular profiles, which displayed irregularly shaped lumina and dysplastic cells. These occasional “nests” of epithelial cells indicated the progression toward malignancy. In a few cases, biliary ducts with more extreme disorganization of the epithelial cells were noticed (Figure 3). However, these cells were not sufficiently dysplastic to sustain a diagnosis of established cholangiocarcinomas. Infiltrations of pigmented MACs were often encountered as aggregates of various sizes, which were either located in close proximity to the bile ducts in areas with bile duct hyperplasia or primarily in the stroma (Figure 4).

Parasites (presumably plasmodia of unidentified myxosporean species) were observed on the outside of the liver (attached to the liver capsule) or inside the organ (primarily in the lumen of the biliary channels) (Figure 5). Anatomical changes in the biliary ducts near to where the parasites were attached were primarily confined to the epithelium. In such cases,

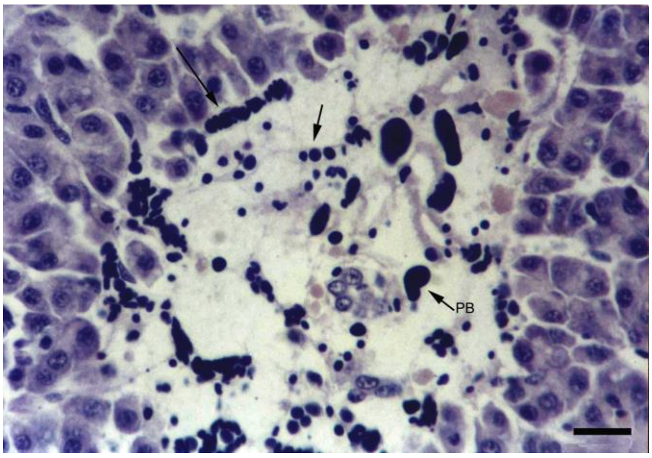

Figure 2. Light micrograph of the Ohrid trout liver. This particular necrotic focus has numerous small roundish basophilic bodies (arrows), as well as larger basophilic and pleomorphic bodies (PB). This image further shows a focus of cholangiocyte death, in which a destroyed biliary channel is still identifiable (arrow). H&E. Scale bar = 20 µm.

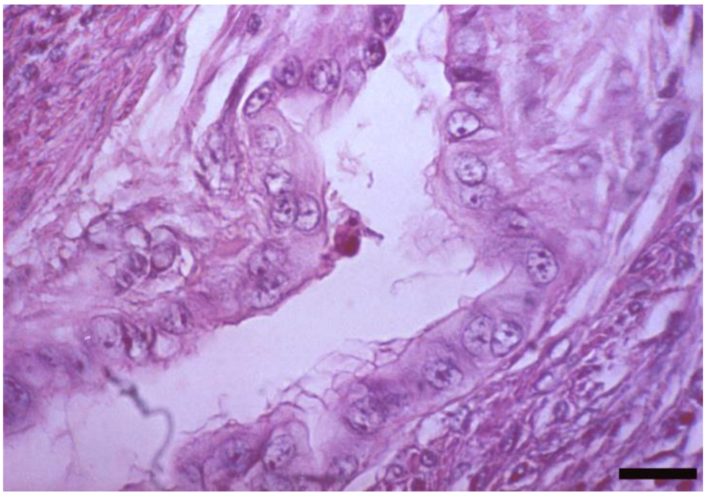

Figure 3. Light micrograph of the Ohrid trout liver. This image shows a biliary duct with dysplastic changes (irregular, distorted profile, abnormally increased nuclear and cellular polymorphism of the epithelial cells). H&E. Scale bar = 20 μm.

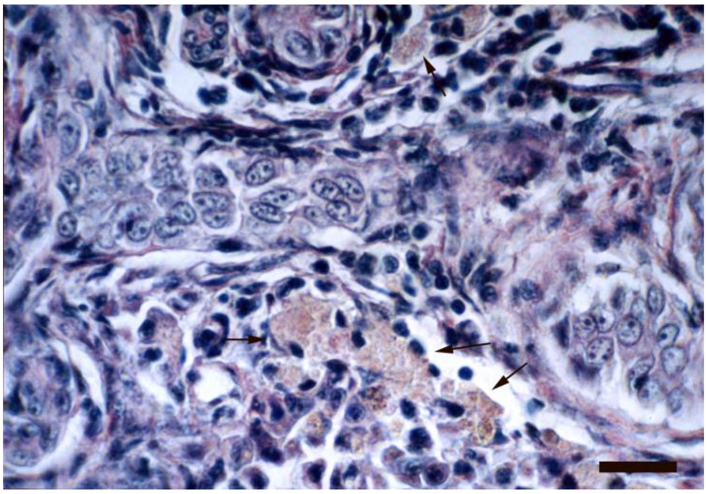

Figure 4. Light micrograph of the Ohrid trout liver. This image shows pigmented macrophages (arrows) close to tangentially sectioned bile ducts, with dysplastic cells (asterisks). H&E. Scale bar = 20 μm.

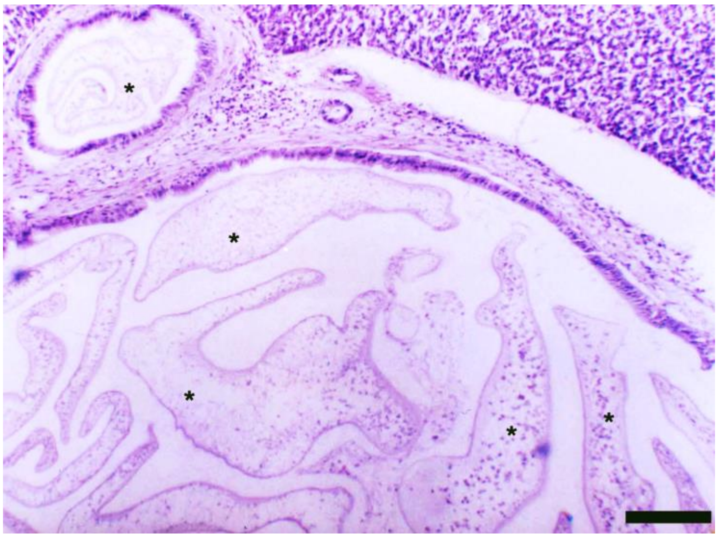

Figure 5. Light micrograph of the Ohrid trout liver. This image shows bile ducts containing parasites (asterisks) that are freely floating in the lumen. H&E. Scale bar = 100 μm.

the epithelium either showed hyperplasia or became more cuboidal (instead of columnar). Among the epithelial cells, maturing rodlet cells were occasionally present (not in all fish). The stromal tracts of ducts infected with parasites sometimes exhibited an increase in the associated connective tissue and, less frequently, lymphocyte infiltration. In this type of changed connective tissue, a large number of mast cells/eosinophilic granule cells were also occasionally present.

The overall and site-based percentage of fish with specific lesions is presented in Table 2. There were no significant differences in the occurrence of fish with specific lesions among the 3 sampling sites. When considering all lesions combined, the percentage of affected fish seemed to decline from the urban site of Andon Dukov-Virce (40%), toward the urban embayment of Pestani (27%), and towards the less urban embayment of Veli Dab-Trpejca (7%). In agreement, a significant difference was obtained between Andon Dukov-Virce and Veli Dab-Trpejca.

The relative volume of hepatic MACs is presented in Table 3, with respect to sampling location and the liver condition of the fish (i.e., with or without liver lesions). Fish with lesions from Pestani appeared to have higher relative MAC volume (~ 3 times, in average) compared to fish with lesions from the other 2 locations; however, no statistically significant difference was proved. However, the data suggest a significantly positive relationship between the presence of lesions and the relative volume of MACs at Pestani. At this site, the mean MAC volume was significantly higher (~ 5 times) in the fish with lesions compared to fish without lesions. Finally, considering all sites combined, the MACs' volume was significantly greater in fish with lesions.

Discussion

The hepatic lesions found in the fish in this study were divided into groups according to their relative value as indicators of contaminant exposure (Mayer *et al.*, 1992; Hinton *et al.,* 1992; Hinton 1993). Lymphocyte infiltration, death (viz. by necrosis), connective tissue proliferation, and parasite infection all are non-neoplastic in nature, and may be related to environmental pollution. For example, lymphocyte accumulation has been detected in fish exposed to organic contaminants (Mayer *et al.*, 1992), heavy metals (Schmidt *et al.*, 1999), pesticides, and other types of pollutants (Rousseaux, Braunchaud, and Spear 1995). However, it is not just toxicants that cause lymphocyte accumulations to emerge, with parasites also having a similar effect. Apropos, myxozoan and other parasitic infections might indirectly be linked to anthropogenic pollution. This occurs not only because of increased susceptibilities of the final host fish, via pollution-induced immune depression (Duneir, 1996; Segner *et al.*, 2012), but also because some hosts for the fish-infecting stages, viz. tubificid oligochaetes, are pollution-tolerant and can be over-represented in urban outflow polluted areas (Carr and Hiltunen, 1965; Milbrink 1983). We also could not assign pericholangiolar fibrosis or focal necrosis in the liver to specific agents (Schmidt-Posthaus *et al.*, 2001), as these phenomena may be induced by a

Table 2. Sample prevalence (percentage of affected Ohrid trout) at the 3 study sites in Lake Ohrid

Geographic Areas (sites)	*Lesion types*					Total
	LyL	Ne	ChF	BiHR	Pa	
Andon Dukov – Virce (n = 15)	13	2	27	27	13	40[a]
Pestani (n = 41)	22	12	20	15	5	27
Veli Dab – Trpejca (n = 14)	7	0	7	7	7	7[b]
All Sites (n = 70)	17	11	16	13	7	64

Abbreviations: LyL, lymphocytic infiltration; Ne, necrosis; ChF, cholangiofibrosis; BiHR, bile duct hyperplasia plus regressive changes in biliary epithelial cells; Pa, parasites within bile ducts. In Andon Dukov–Virce, lesions were significantly more frequent (a vs b, p = 0.0294, via the T-test for proportions and p = 0.0378, via the Z-test), relative to Veli Dab-Trpejca (selected as the reference site).

Table 3. Relative volume (V_V) of MACs in the liver of Ohrid trout at the 3 study sites in Lake Ohrid[1]

Geographic Areas (sites)	V_V (MACs/liver) (%)		
	No lesioned liver	*Lesioned liver*	Total
Andon Dukov – Virce (n = 15)	0.13 (1.51)	0.20 (0.67)	0.16 (0.46)
Pestani (n = 41)	0.13 (0.95)[A]	0.62 (1.19)[B]	0.26 (1.70)
Veli Dab – Trpejca (n = 14)	0.15 (0.67)	0.20 (n.a.)	0.16 (0.01)
All Sites (n = 70)	0.14 (0.80)[A]	0.46 (1.34)[B]	0.22 (1.58)

[1] Values are expressed as means (coefficient of variation). [2] Data not available (n.a.) because n = 1. Different uppercase subscript letters represent differences between sampling sites regarding fish with and without liver lesions (read horizontally), according to the Students T-test; A versus B, P<0.0001.

range of different chemical stressors, including heavy metals (Mallatt 1985). High concentrations of heavy metals in Ohrid trout, in addition to other fish species from Lake Ohrid, have been previously reported using histochemical (Jordanova 2004) and chemical (Spirkovski 2001) methods, indicating the potential negative effects of metal pollution on fish in this lake. While heavy metals are likely to be one major cause of pathological changes in Ohrid fish, other parameters are also likely to be involved.

In contrast, bile duct hyperplasia and biliary epithelial cell dysplasia are recognized as being closely linked with pollution for a variety of species (Mayer *et al.*, 1992; Schmidt *et al.*, 1999; Murchelano and Wolke 1991; Rousseaux *et al.*, 1995; Stentiford *et al.*, 2003). Both conditions are now well established as histopathological biomarkers of contaminant exposure, in both field and laboratory studies (Mayer *et al.*, 1992; Schmidt *et al.*, 1999). In fishes from Lake Ohrid, bile duct proliferation has been previously reported for the Ohrid minnow moranec, *Pachycilon pictus* (Roganovic-Zafirova and Tavcioska-Vasileva 1998/99), and in virtually every collected black barbel, *Barbus meridionalis petenyi* (Roganovic-Zafirova *et al.*, 2001/02). These two species showed a 75–97% prevalence of the cholangioproliferative lesions. In comparison, the frequency of cholangiofibrosis (13%) and bile duct hyperplasia at all 3 sites, plus regressive changes in biliary epithelial cells (16%) obtained in the current study seemed relatively low. Regardless of the known or speculated (e.g., pollution exposure over time) causes of such disorders, this type of lesion typically results from chronic exposure to environmental contamination. These types of lesions are chronic in wild fish exposed to chemically polluted sites (Murchelano and Wolke 1991).

The frequency of pathological findings varied slightly among the 3 sampling sites. However, fish from the more urban areas tended to exhibit greater pathological changes, which is consistent with existing assumptions and some information about the low water quality in these areas. Contaminants in the water cause organic alterations, which may compromise fitness, reproduction, and/or even cause death, ultimately leading to population declines (Bucke and Feist 1993; Schmidt-Posthamus *et al.*, 2001). The lesions recorded in the present study, particularly bile duct abnormalities, suggest that stressors (probably pollutants) are present in Lake Ohrid; hence, these stressors might be factors contributing to the population decline of Ohrid trout in recent decades. In addition, the results of this study are compatible with the presence of a pollution gradient (pattern) along the eastern coast of the lake; with decreasing pollution towards the south. This idea nicely agrees with studies that investigated gastropods, which found that while in areas close to the town of Ohrid (station Virce) pollution tolerant species existed, in locations near Veli Dab species typical of cleaner waters predominated (Stankovic-Jovanovic, 2007). Our pathological findings are in accordance as well with recent data from chemical monitoring of organochlorine pesticides in both sediment and water (Veljanoska-Sarafiloska *et al.*, 2011), which showed higher concentrations at the north-eastern more costal part of the Lake (roughly halfway between Andon Ducov and near Virce, subject to urban inputs from both the town of Ohrid and Andon Dukov campsite), and lower amounts at the south-eastern part of the Lake (close to Veli Dab). Also agreeing with the present data and our suggestion of a pollution gradient is a recent palaeolimnological study on the anthropogenic impact in the lake, which concluded that “living conditions for the endemic species in Lake Ohrid have become less favorable in the northern part of the lake” (Lorenschat *et al.*, 2014). Therefore, in view of our indirect evidences — which should be looked at as exploratory research — and of recent data, further studies are required to confirm the presence of pollution gradients and to identify the most polluted areas of Lake Ohrid.

Over 60 published (qualitative or semi-quantitative) histopathological studies on various fish species proposed that environmental contamination causes an increase in MAC fluctuations (size, number, and/or pigment composition) (e.g., Blazer *et al.*, 1994; Blazer, Fournie, and Weeks-Perkins, 1997; Krüger *et al.*, 1996; Elston *et al.*, 1997; Meinelt *et al.*, 1997; Facey *et al.*, 1998; O’Farrell, Elliott, and Landolt 2000; Fournie *et al.*, 2001). Such association is in line with response of MACs in controlled contaminant experiments. For instance, proliferation-growth of MACs were noted after exposure to: arsenic (Blazer, Fournie, and Weeks-Perkins, 1997); diazinon (Dutta *et al.*, 1997); copper, iron and zinc enriched diets (Manera *et al.*, 2000); perchlorate (Capps *et al.*, 2004); dietary Pb(II) (Rabitto *et al.*, 2005); pulp mill effluent (van den Heuvel *et al.*, 2005); and cadmium chloride (Suresh 2009). The current study is also consistent with this association, because the mean values of the relative MAC volume were 1.5 to 4.7 times greater in fish with lesioned livers (Table 3). Overall, pigmented MACs increased in fish with liver lesions; with significant correlations markedly demonstrated in the Pestani area and when considering all the sites. In addition to environmental contamination, changes in MACs may be related to certain endogenous factors, such as gender, age, size, and nutritional status (Agius and Agbede 1984; Krüger *et al.*, 1996; Blazer *et al.*, 1997; Rocha, Monteiro, and Pereira 1997; Agius and Roberts 2003). In addition, MACs production seems to be related to reproductive stage (Krüger *et al.*, 1996; Elston *et al.*, 1997; Jordanova 2004; Jordanova, Miteva, and Rocha 2008). However, while only female fish were sampled in this study, the fish were fairly comparable among sites in size and weight; therefore, observed differences (Table 3) were most probably not caused primarily by endogenous factors. The MACs observed in fish from Pestani appeared to be linked to the lesions/stressors, because animals with liver lesions collected from this site had a significantly lower CF compared with those without lesions; being the CF often decreased under the presence of pollutants (Di Giulio and Hinton 2008). However, the percentage of single specific lesions was not statistically associated with sampling location. It is possible that the quantity of MACs influences the occurrence of lesions, but with other factors also contributing. For instance, some non-specific immune functions of salmonids are believed to become diminished, or even impaired, as the fish reach sexual maturity (Alcorn, Murray, and Pascho 2002). As well, female Ohrid trout liver MACs proliferate soon after spawning (Jordanova, Miteva, and Rocha 2008), but that fact unlikely impacted herein, as the analyzed fish were not post spawning individuals.

In conclusion, although the number of fish assessed in this study was limited, the results suggest the presence of some unspecific toxicopathic lesions in the liver of Ohrid trout. This observation lends support to the theory that this species is being exposed to different levels of xenobiotics at various locations along the lake's shoreline. The studied endpoints indicated that Veli Dab-Trpejca had the lowest levels of pollution and biological impacts compared to the other more urban sites; this is perfectly in line with what we hypothesize facing the positioning of the sampling locations, and water quality based on *E. coli* levels (LOCP, 2002). The timeliness of our findings are supported by more recent data (Stankovic-Jovanovic, 2007, Veljanoska-Sarafiloska *et al.*, 2011, Lorenschat *et al.*, 2014). One uncontrolled confounding factor herein is that we cannot prove that the fish were living only in the areas from which they were collected. Despite there are no natural or man-made barriers, movements of the fish across the Lake remain virtually unstudied. Despite all the limitations, to monitor changes in water quality in Lake Ohrid over time, baseline reference quantitative information, such as that presented in this study, is of very high value. It is likely that Ohrid trout have been subject to long-term exposure to pollutants (extending over at least 20 years since urban development and industry were established in this region); therefore, overfishing should not be viewed as the only or primary cause of the population decline of Ohrid trout. Chemical screening and studies with a broad biomarker and bioindicator panel should be established, with this study evidencing the utility of the Ohrid trout as a potential bioindicator.

Acknowledgments

The authors thank M.Sc. Zoran Spirkovski of the Hydrobiological Institute, Ohrid, Macedonia, for helping in the collection of fish and for fieldwork assistance. We further acknowledge the generous and efficient support of the mentioned Hydrobiological Institute during the fieldwork.

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