UNIVERSITY OF NOVA GORICA
SCHOOL OF ENVIRONMENTAL SCIENCES

OXIDATIVE STRESS IN FISH ORGANS

DIPLOMA THESIS

Jelena Topić

Mentor: Assist. Prof. Dr. Andreja Ramšak

Nova Gorica, 2013

STATEMENT

I declare that this bachelor thesis is a result of my own research work. Results which have been created by other researchers either in collaboration or separately are properly cited in the thesis.

Jelena Topić

ACKNOWLEDGMENTS

The experimental work for the Bachelor thesis was carried out at the University of Trás-os-Montes and Alto Douro (UTAD) under the supervision of Dr. Ana Maria Coimbra and Dr. Sandra Mariza Veiga Monteiro. The work was performed within the project Biomonitoring in rivers: an integrated approach (PTCD/BIA-ECS/114859/2009) and has been co-founded by COMPETE program, QREN program and European funds. Travel costs were covered by the Erasmus scholarship in the period from February 2012 to July 2012.

Firstly, I would like to acknowledge and thank my supervisor Assist. Prof. Dr. Andreja Ramšak and the supervisors at UTAD, Dr. Ana Maria Coimbra and Dr. Sandra Mariza Veiga Monteiro for all the help, guidance, advice and recommendations during the course of the investigation and after it. I would also like to thank Sandra Pereira who assisted me, provided all the necessary information, and had the patience to answer all of my questions regarding practical work.

I would like to give my sincere thanks to Estera and Špela for being wonderful and supportive friends. Estera you are amazing and I am forever grateful for all the effort you invested in the proof-reading of this thesis. A girl could not ask for better friends.

Last but not least, the biggest thank you goes to my family for guidance and for supporting my ambitions, morally as well as financially.

ABSTRACT

Reactive oxygen species are a crucial part of oxidative stress. Important sources of reactive oxygen species are chemical pollutants, i.e. pollutants that are capable of redox cycling. In order to determine pollution in the river basins of Ave, Cávado and Douro, fish species abundant in those areas were used. Specimens of the genus *Pseudochondrostoma* and *Luciobarbus bocagei* were captured from nine sampling sites with different ecological status. I used gills of these species to determine lipid peroxidation (LPO), glutathione-*S*-transferase (GST) and superoxide dismutase (SOD) as biomarkers of exposure. Values of lipid peroxidation have shown no statistical difference ($p>0.05$) among sites in both species. The values of LPO were in both species higher in the summer season and lower in the winter season. GST levels were higher in both species in polluted sites, and in *Pseudochondrostoma* sp. seasonal variation was observed, as GST activity was higher in the summer season in comparison to the winter season. Results of GST enzyme activity in *Pseudochondrostoma* sp. have shown statistical difference in the summer in the site Caldas Vizela (bad ecological status) when comparing it to other sites. In *L. bocagei* statistical difference was observed in the summer samples, as GST enzyme activity showed an increase by four times in the site Caldas Vizela when comparing it to the site Vizela Sto. Adrião (poor ecological status). SOD activity was determined only in the winter samples of *L. bocagei*. SOD activity was low in all samples and there was no statistical difference between sites. The most contaminated site showed to be Caldas Vizela since the site had the highest GST enzyme activities in both *L. bocagei* and *Pseudochondrostoma* sp. in the summer season. In the winter season, it was observed that in *Pseudochondrostoma* sp. GST enzyme activities were also the highest in Caldas Vizela, which is consistent with the Water Framework Directive classification of the site (bad ecological status).

Key words: biomarkers of oxidative stress, freshwater fish, *Pseudochondrostoma* sp., *Luciobarbus bocagei*, Ave, Cávado and Douro river basins

POVZETEK

Reaktivne kisikove spojine so ključen del oksidativnega stresa. Pomemben vir reaktivnih kisikovih spojin so kemijski onesnaževalci, predvsem onesnaževalci, ki so sposobni redoks reakcij. Za ugotavljanje onesnaženosti v porečjih rek Ave, Cávado in Douro smo izbrali vrste rib, ki so na teh območjih pogoste. Osebki iz vrst *Pseudochondrostoma* sp. in *Luciobarbus bocagei* so bili ulovljeni na devetih vzorčnih mestih z različnim ekološkim stanjem. Škrge obeh vrst so bile uporabljene za določitev peroksidacije lipidov (LPO), aktivnosti glutation-*S*-transferaze (GST) in aktivnosti superoksid dismutaze (SOD) kot biomarkerjev izpostavljenosti oksidativnemu stresu. Vrednosti LPO obeh vrst niso pokazale statistične razlike ($p>0.05$) med vzorčnimi mesti. Vrednosti LPO so bile višje v poletnem času v primerjavi z zimskih časom pri obeh vrstah. Aktivnost encima GST je bila v obeh vrstah višja na onesnaženih vzorčnih mestih. Pri *Pseudochondrostoma* sp. je bila vidna sezonska variabilnost, saj so bile vrednosti encima višje v poletnem času in nižje v zimskem času. Statistična razlika je bila opažena v poletnem času med vzorčnim mestom Caldas Vizela (slabo ekološko stanje) in ostalimi vzročnimi mesti. Pri *L. bocagei* pa je bila statistična razlika opažena v poletnem času med vzorčnima mestoma Caldas Vizela in Vizela Sto. Adrião (slabše ekološko stanje). Na vzorčnem mestu Caldas Vizela je bila vrednost aktivnosti GST štirikrat višja kot na mestu Vizela Sto. Adrião. Aktivnost encima SOD je bila določena le v zimskih vzorcih pri vrsti *L. bocagei*. Aktivnost je bila nizka na vseh vzorčnih mestih, statistične razlike med mesti pa ni bilo. Rezultati encimskih aktivnosti kažejo, da je najbolj onesnaženo vzorčno mesto Caldas Vizela, saj so bile na tem vzorčnem mestu poleti najvišje vrednosti encima GST pri obeh vrstah. Iz zimskih vzorcev je razvidno, da so bile pri *Pseudochondrostoma* sp. vrednosti encima GST prav tako najvišje na mestu Caldas Vizela, kar je tudi v skladu s tem, da je ekološko stanje tega mesta na podlagi Vodne direktive označeno kot slabo.

Ključne besede: biomarkerji oksidativnega stresa, sladkovodne ribe, *Pseudochondrostoma* sp*., Luciobarbus bocagei*, porečja rek Ave, Cávado in Douro

Obsežen povzetek v slovenščini

Oksidativni stres v organih rib

Diplomsko delo

TEORETIČNE OSNOVE

V zadnjem stoletju smo bili priča globalnemu industrijskemu razvoju, ki je veliko pripomogel k onesnaževanju zraka, zemlje in voda. S povečevanjem človeških aktivnosti se v okolje sprošča vse več kemikalij in veliko teh kemikalij predstavlja grožnjo organizmom in ekosistemom (Henzová in sod., 2008).

Povečana proizvodnja in uporaba pesticidov v kmetijstvu za zaščito rastlin in živali je privedla do onesnaženja površinskih in podzemnih voda. Nekatere izmed teh snovi so težko razgradljive in so tudi toksične, mutagene in karcinogene (Aksu, 2005).

Vodni ekosistem je, tako kot ostali ekosistemi, izpostavljen toksičnim onesnaževalom, ki nastajajo pri industrijskih, kmetijskih in gospodinjskih aktivnostih (Oliveira et al., 2010). Največje težave predstavljajo izpusti težkih kovin v sladkovodnih in morskih ekosistemih. Za izpuste so odgovorne industrijske odpadne vode, petrokemijska, jeklarska in avtomobilska industrija ter obrati, ki se ukvarjajo z izdelavo gnojil (Henzová in sod., 2008).

Za izboljšanje stanja ekosistemov je potrebno vedeti katera vodna telesa so prizadeta in kateri faktorji so povzročili poslabšanje stanja ekosistema. Temelj monitoringa so navadno fizikalne, kemijske in bakteriološke meritve, saj nam dajo podatke, ki so potrebni za ustrezno in pravilno gospodarjenje z vodami (Metcalfe, 1989).

Kljub temu, da ima kemijsko onesnaženje primarno vlogo, zgolj kemijske analize v sklopu okoljskega monitoringa pri določevanju negativnih učinkov, ki jih povzročajo kompleksne mešanice kemikalij na onesnaženih področjih, ne bodo dovolj (Ozmen in sod., 2008). To je še posebej vidno v tekočih vodah, kjer so spremembe v hidrologiji hitre in zato težko določljive. Kemijske meritve ne morejo odražati integracije okoljskih faktorjev in dolgoročne trajnosti vodnih ekosistemov zaradi njihove hitre spremenljivosti (Li in sod., 2010).

Koncentracije onesnaževal v površinskih vodah so navadno nizke, vendar so onesnaževala po navadi prisotna skozi daljša časovna obdobja. Pod temi pogoji imajo lahko substance kronične učinke. Učinek teh substanc ne privede do smrti prizadetih organizmov, bolj pogosto pride do okvare pomembnih funkcij v organizmu, kar privede do manjše vitalnosti organizmov (Soldán, 2003).

Biološki monitoring ali biomonitoring se je izkazal za nujen in učinkovit dodatek k tradicionalnim tehnikam monitoringa (Soininen & Könönen, 2004). Biološki monitoring uporablja organizme za detekcijo onesnaženosti okolja, saj lahko onesnaženje sproži negativne učinke v živalih na različnih strukturnih nivojih (nivo celic, tkiv in organov), lahko pa pride tudi do prilagoditve živali na spremembe. Takšne reakcije so kompleksne in odvisne od več faktorjev, med njimi tudi od tega kakšno onesnaževalo je prisotno in kakšna je njegova koncentracija, od stopnje izpostavljenosti ter od dovzetnosti vsakega organa posebej. Še en pomemben faktor, ki prispeva h končnemu

toksičnemu učinku, so sinergistične ali antagonistične interakcije med onesnaževali (Fenoglio in sod., 2005).

Odgovori na onesnaževanje se navadno odražajo kot spremembe v aktivnosti nekaterih encimov, še posebej v ključnih encimih, ki sodelujejo v biotransformacijskih sistemih organizmov Te spremembe v aktivnosti encimov lahko uporabimo kot biomarkerje in le-ti nudijo specifično orodje pri določanju učinkov onesnaževal (Ozmen in sod., 2008).

Ribe so najpogosteje uporabljen vretenčarski model v ekotoksikoloških študijah (Fernandes in sod., 2008) zaradi njihove občutljivosti na onesnaženje in posebnih bioloških lastnosti (relativno velika velikost telesa, dolg življenjski cikel). Poleg tega so ribe na vrhu prehranjevalne verige in vir hrane za človeka, kar pomeni, da obstaja možnost za njihov neposreden vpliv na človekovo zdravje (Zhou in sod., 2008). Pomembno je vedeti, da imajo lahko starost, spol in aktivnost pomemben vpliv. V krvi je težko določiti onesnaževala, kar pomeni, da je za določanje stopnje onesnaženja potrebno uporabiti različne organe, najpogosteje škrge, jetra, ledvice, mišice ali kar celotno telo (van der Oost in sod., 2003).

Škrge imajo osrednjo vlogo v fiziološkem ravnovesju, saj tu poteka izmenjava plinov, osmotska in ionska regulacija, kislinsko-bazno ravnotežje in izločanje dušika (Ahmad in sod., 2008; Monteiro in sod., 2009). Škrge so prva stična točka med onesnaževali prisotnimi v vodi in ribo. Nizke koncentracije kovinskih ionov vplivajo na izmenjavo ionov v epiteliju škrg, višje koncentracije pa lahko povzročijo poškodbe škrg in akumulacijo sluzi (Playle, 1998).

Biološki markerji ali biomarkerji so kemijsko inducirane spremembe, ki so merljive v organizmu. Merimo jih lahko v telesnih tekočinah, celicah ali tkivih in so indikatorji prisotnosti in razsežnosti toksičnih substanc ali odgovora organizma. Dobri biomarkerji so indikatorji biorazpoložljivosti onesnaževal in zgodnjih bioloških odzivov ter se lahko uporabijo po izpostavljenosti organizma prehranskim in okoljskim virom onesnaženja. Uporabljajo se pri določevanju vzročno-posledičnih povezav in povezav med odmerkom in učinkom z namenom ocene tveganja v kliničnih študijah in za potrebe monitoringa (Suter, 1990; van der Oost in sod., 2003). Biomarkerji v ribah so lahko uporabno orodje v monitoringu kvalitete vodnih ekosistemov (van der Oost in sod., 2003).

Lipidna peroksidacija (LPO) je oksidacija polinenasičenih maščobnih kislin, ki so občutljive na oksidativne reakcije kisikovih reaktivnih spojin zaradi njihovih dvojnih vezi in je pomembna posledica oksidativnega stresa (van der Oost in sod., 2003).

Superoksid dismutaza (SOD) pripada skupini metaloencimov, ki so odgovorni za katalizo pretvorbe reaktivnih superoksidnih anionov ($O_2^{-\bullet}$), pri čemer nastane vodikov peroksid (H_2O_2), ki je pomembna reaktivna kisikova zvrst (van der Oost in sod., 2003). SOD igra ključno antioksidativno vlogo, saj se je njegova pomembnost pokazala v vseh preučevanih aerobnih organizmih (Stegeman in sod., 1992; op cit: van der Oost in sod., 2003).

Glutation-*S*-transferaza (GST) spada med encime, ki katalizirajo konjugacijo elektrofilnih spojin do glutationa (GSH). GST je tripeptidni protein, ki je sestavljen iz treh enot (Chubben in sod, 2001). Poleg pomembne funkcije v znotrajceličnih transportnih sistemih prispevajo k obrambi zoper oksidativni stres, saj imajo ti encimi

sposobnost detoksifikacije škodljivih endogenih substanc (Tew, 1994; Chubben in sod., 2001).

CILJI DIPLOMSKEGA DELA

- Ovrednotenje učinkov onesnaženja na vzorčnih mestih z različnim ekološkim statusom z uporabo biomarkerjev izpostavljenosti stresu;
- ovrednotenje biomarkerjev izpostavljenosti (oksidativnemu stresu in celičnih poškodb) v ribah *Pseudochondrostoma* sp. in *Luciobarbus bocagei*:
    - ovrednotenje lipidne peroksidacije;
    - aktivnost glutation-*S*-transferaze;
    - aktivnost superoksid dismutaze;
- primerjava rezultatov med vrstami, med različnimi vzorčnimi mesti in med letnima časoma (poletje in zima).

PRAKTIČNI DEL

Vzorčenje

Vzorčenje je potekalo poleti leta 2011 in pozimi leta 2012. Vzorčene so bile ribe rodu *Pseudochondrostoma* (Robalo, Almada, Levy and Doadrio 2007; Cyprinidae: Leuciscinae) in vrsta *Luciobarbus bocagei* (Steindachner, 1864; Cyprinidae: Barbinae*),* ki so bile ulovljene na vzorčnih mestih z različno stopnjo onesnaženosti v porečjih rek Ave, Cávado in Douro. Vzorčna mesta so bila izbrana glede na geografsko razširjenost vrst v glavnih strugah, z upoštevanjem različnih virov in tipov onesnaženja in glede na referenčna mesta. Vzorčna mesta so bila: Ponte de Pingue (dobro ekološko stanje), Prado (slabše ekološko stanje), Ponte da Junqueira (slabše ekološko stanje), Ponte Trofa (slabše ekološko stanje), Vizela Santo Adrião (slabše ekološko stanje), Tejão (dobro ekološko stanje), Pinhão (dobro ekološko stanje), Graça (slabo ekološko stanje) in Caldas de Vizela (slabo ekološko stanje). Mesta z dobrim ekološkim stanjem so bila referenčna mesta.

Pseudochondrostoma (Cyprinidae) je endemičen rod na Iberskem polotoku in vanj uvrščamo tri vrste: *P. duriense* (Coelho, 1985), *P. polylepis* (Steindachner, 1864) in *P. wilkommii* (Steindachner, 1866). Vse tri vrste živijo v srednjih delih rek na območjih s hitrejšim tokom. Hranijo se z majhnimi nevretenčarji, odmrlimi snovmi in rastlinjem na dnu rek. Vse tri vrste se razmnožujejo spomladi (Kottelat and Freyhof, 2007).

Luciobarbus bocagei (Cyprinidae) živi v srednjih in spodnjih delih rek na območjih z nizkim tokom. Vrsto najdemo v Španiji in na Portugalskem. Prehranjujejo se predvsem z bentoškimi nevretenčarji in odmrlo snovjo. Razmnožujejo se v obdobju pozno spomladi do zgodnjega poletja (Kottelat and Freyhof, 2007).

Priprava tkiva za encimske teste

Ribe so bile ulovljene in nato uspavane s potopitvijo v uspavalo MS-222 (m-aminobenzoat metasulfonske kisline) ter zmerjene in stehtane. MS-222 je uspavalo ki se uporablja kot pomirjevalno sredstvo in za evtanazijo poikilotermnih organizmov (Cakir & Strauch, 2005). Sledilo je obglavljenje rib, škrge so bile odstranjene ter zamrznjene v tekočem dušiku in nato shranjene na - 80°C.

V laboratoriju sem škrge homogenizirala v homogenizacijskem pufru s Potter-Elvenhjem homogenizatorjem. Po homogenizaciji je sledilo centrifugiranje pri 15 000 rpm in pri 4°C. Po 20 minutah centrifugiranja sem pobrala supernatant, ki smo ga uporabili za določanje vsebnosti proteinov in za meritve LPO ter aktivnosti SOD in GST.

Lipidna peroksidacija (LPO)

Uporabili smo metodo TBARS (Niki, 2000), ki smo jo priredili za mikrotitersko ploščico. Supernatantu smo dodali TCA (trikloroocetno kislino), nato pa vorteksirali in centrifugali 20 minut pri 5000 rpm. Po centrifugiranju smo znova pobrali supernatant in dodali 0,1 M EDTA ter raztopino A (1 % TBA /tiobarbiturna kislina/, 0,05 M NaOH in 0,025 % BHT /butilhidroksi toluen/) in segrevali 30 minut v vodni kopeli. Po segretju smo počakali, da se vzorci ohladijo na sobni temperaturi. Po ohladitvi smo vzorce v duplikatih prenesli v mikrotitersko ploščo in določili absorbanco pri 532 nm. Prav tako smo določili absorbanco kontrolnega vzorca. Določali smo vsebnost MDA, saj pri reakciji TBA ena molekula MDA reagira z dvema molekulama TBA, pri čemer se tvori pigment, ki ima absorpcijski maksimum med 532 in 535 nm.

Določanje vsebnosti proteinov

Vsebnost proteinov smo določili z metodo po Lowryju (Lowry, 1951). Pripravili smo standardne raztopine z različno koncentracijo BSA (goveji serumski albumin). V mikrotitersko ploščo smo nanesli vzorce skupaj z destilirano vodo v duplikatih. V njo smo nanesli tudi standardne raztopine. Nato smo v vsak vzorec dodali tudi mešanico raztopin (raztopina A+B). Ploščo smo inkubirali 10 minut in po inkubaciji dodali Folinov reagent v razmerju 1:1. Nato smo inkubirali še enkrat. Inkubacija je potekala 30 minut in po končani inkubaciji smo določili absorbanco pri valovni dolžini 690 nm.

Določanje aktivnosti GST

Aktivnost GST je bila določena po metodi, ki so jo razvili Habig in sod. (1974) in prirejena za mikrotitersko ploščo po postopku, ki sta ga razvila Frasco in Guilhermino (2002). Aktivnost GST je izračunana kot razlika v absorbanci med kontrolnim vzorcem ter med reaktivno mešanico in vzorcem po celotni encimski pretvorbi substrata v produkt. Najprej sem pripravila potrebne raztopine. Uporabili smo 0,1 mL supernatanatanta in 0,2 mL reaktivne mešanice. GST aktivnost smo določili takoj pri 340 nm. Meritev je potekala 5 minut in v 20 sekundnih intervalih. Vse reakcije so bile izvedene v duplikatih.

Določanje aktivnosti SOD

Aktivnost encima SOD je bila določena po metodi McCord in Fridovich (1969) in prirejena za mikrotitersko ploščo. Glede na koncentracijo proteinov v supernatantu smo redčili vzorce do 4 mg/mL proteinov. Pripravili smo potrebne raztopine za določitev aktivnosti SOD in standardne raztopine SOD z različno koncentracijo SOD (koncentracija SOD je bila izražena v U encima SOD na mL). Absorbanco smo merili 2 minuti pri valovni dolžini 550 nm, v 20 sekundnih intervalih in nato izračunali aktivnost SOD.

Statistična analiza

Rezultati so izraženi kot aritmetična sredina ± standardna napaka aritmetične sredine. Statistična analiza je bila izvedena z analizo variance (enosmerna ANOVA), ki ji je sledil Tukey test. Odzivi so bili ovrednoteni kot statistično različni v primerih, kjer je p vrednost znašala manj kot 0,05.

REZULTATI IN DISKUSIJA

Lipidna peroksidacija (LPO)

LPO smo določali v osebkih *Pseudochondrostoma* sp. in *L. bocagei* v poletnem (2011) in zimskem (2012) letnem času.

V poletnem času so bili osebki *Pseudochondrostoma* sp. vzorčeni na petih različnih mestih: Tejão, Pinhão, Prado, Vizela Sto. Adrião in Caldas Vizela. Vsebnosti MDA so bile pri *Pseudochondrostoma* sp. v poletnem času višje na referenčnih mestih Tejão (23,88 ± 10,86 nmol MDA/mg protein) in Pinhão (39,36 ± 29,76 nmol MDA/mg protein). Vrednosti MDA so bile nižje na onesnaženih mestih Prado (5,99 ± 1,05 nmol MDA/ mg protein), Vizela Sto. Adrião (27,13 ± 9,49 nmol MDA/ mg protein) in Caldas Vizela (9,31 ± 2,62 nmol MDA/mg protein), ki je glede na Vodno direktivo najbolj onesnaženo mesto. Ne glede na to, med vzorčnimi mesti ni bilo statistične razlike ($p>0,05$). Pri interpretaciji rezultatov z vzorčnega mesta Pinhão je potrebno upoštevati, da sta bila na voljo le dva vzorca, in da je bila velika razlika v vrednosti MDA med tema vzorcema.

Pozimi 2012 so bili osebki *Pseudochondrostoma* sp. vzorčeni na treh mestih: Ponte Pingue, Prado in Caldas Vizela. Vsebnost MDA je bila najnižja na referenčnem mestu Ponte Pingue (7,02 ± 1,25 nmol MDA/ mg protein), na onesnaženem mestu Caldas Vizela so bile vrednosti višje (9,46 ± 3,64 nmol MDA/ mg protein), kar je bilo pričakovano. Odzivi med vzorčnimi mesti niso bili signifikantno različni ($p>0,05$).

Osebki *L. bocagei* so bili vzorčeni poleti 2011 na štirih različnih mestih: Ponte Junqueira, Vizela Sto. Adrião, Graça in Caldas Vizela. Vsebnosti MDA so bile najvišje na mestu Ponte Junqueira (56,48 ± 18,83 nmol MDA/ mg protein). LPO je bila na ostalih vzorčnih mestih znatno nižja, najnižja pa je bila na mestu Caldas Vizela (13,70 ± 6,13 nmol MDA/ mg protein). Statistična razlika je bila med mestom Ponte Junqueira in mestoma Graça in Caldas Vizela. Na mestu Ponte Junqueira so bile vrednosti okoli 5-krat višje kot na mestih Graça in Caldas Vizela. Iz referenčnih mest vzorci niso bili pridobljeni.

Pozimi 2012 so osebke *L. bocagei* vzorčili na petih mestih: Prado, Trofa, Ponte Junqueira, Graça in Caldas Vizela. Vrednosti LPO so bile najvišje na mestih Prado (15,66 ± 7,00 nmol MDA/ mg protein) in Trofa (15,52 ± 6,94 nmol MDA/ mg protein). Na mestu Ponte Junqueira so bile MDA vrednosti (12,47 ± 3,94 nmol MDA/ mg protein) manjše kot na mestih Prado in Trofa. Najnižje izmerjene vrednosti so bile na mestih Graça (5,49 ± 1,74 nmol MDA/ mg protein) in Caldas Vizela (5.38 ± 2.41 nmol MDA/ mg protein). Kljub temu, da so bile razlike v vrednostih med vzorčnimi mesti, pa med odzivi ni bilo signifikantne razlike ($p>0,05$).

Primerjava rezultatov je pokazala, da je bila LPO v škrgah višja poleti 2011 v primerjavi z zimo 2012, tako v *Pseudochondrostoma* sp. kot tudi v *L. bocagei*. Različni dejavniki lahko spodbudijo antioksidativno obrambo pri ribah. Med nje lahko štejemo starost,

prehranjevalne navade ter tudi okoljske dejavnike (spremembe v temperaturi, raztopljen kisik in prisotna onesnaževala) (Martínez-Álvarez in sod., 2005; Trenzado in sod., 2006).

Ena od možnih razlag za višje vrednosti LPO v osebkih *L. bocagei* in *Pseudochondrostoma* sp. poleti 2011 je, da je bila temperatura vode odgovorna za povečano polinenasičenje mitohondrijskih membran v ribah in za dvig stopnje mitohondrijskega dihanja, kar je povečalo proizvodnjo ROS in uhajanje protonov in nato še peroksidacijo teh membran (Oliva in sod., 2012). Prav tako je poleti nivo vode nižji, kar pomeni, da je onesnaženje bolj koncentrirano, kar lahko privede do večjega oksidativnega stresa.

Glutation-*S*-transferaza (GST)

GST smo določali v osebkih *Pseudochondrostoma* sp. in *L. bocagei* v poletnem (2011) in zimskem (2012) času.

Osebki *Pseudochondrostoma* sp. so bili poleti 2011 vzorčeni na petih različnih mestih: Tejão, Pinhão, Prado, Vizela Sto. Adrião in Caldas Vizela. Najvišje vrednosti aktivnosti GST so bile izmerjene na mestu Caldas Vizela (78,47 ± 3,44 nmol/min/mg protein), najnižje pa na referenčnih mestih Tejão (36,56 ± 9,61 nmol/min/mg protein) in Pinhão (16,02 ± 14,09 nmol/min/mg protein). Vrednosti na mestih s slabšim ekološkim statusom - Prado in Vizela Sto . Adrião - so bile nižje kot na onesnaženem mestu Caldas Vizela in višje kot na referenčnih mestih. Statistična razlika je bila med mestom Caldas Vizela in ostalimi vzorčnimi mesti. Na mestu Caldas Vizela je bila aktivnost GST približno 4,5-krat višja kot na mestu Pinhão , približno 2,2-krat višja kot na mestih Tejão in Vizela Sto. Adrião ter 1,7-krat višja kot na mestu Prado.

V letu 2012 so bili osebki *Pseudochondrostoma* sp. vzorčeni na treh mestih: Ponte Pingue, Prado in Caldas Vizela. Najnižja vrednost aktivnosti GST je bila na referenčnem mestu Ponte Pingue (36,82 ± 13,90 nmol/min/mg protein), ki mu je sledilo mesto Prado (50,63 ± 6,02 nmol/min/mg protein). Najvišja vrednost GST je bila izmerjena na mestu s slabim ekološkim statusom Caldas Vizela (56,79 ± 10,57 nmol/min/mg protein). Med mesti ni bilo statistične razlike v vrednosti GST aktivnosti ($p>0,05$).

Osebki vrste *L. bocagei* so bili vzorčeni poleti 2011 na štirih različnih mestih: Ponte Junqueira, Vizela Sto. Adrião, Graça in Caldas Vizela. Najvišje vrednosti aktivnosti GST so bile določene na mestih s slabim ekološkim statusom - Graça (33,35 ± 10,94 nmol/min/mg protein) in Caldas Vizela (47,47 ± 11,16 nmol/min/mg protein). Na mestih Ponte Junqueira in Vizela Sto . Adrião so bile vrednosti GST aktivnosti nižje. Značilna statistična razlika ($p<0,05$) je bila med mesti Caldas Vizela in Sto. Adrião (11,68 ± 2,46 nmol/min/mg protein). Aktivnost encima GST je bila na vzorčnem mestu Caldas Vizela približno 4-krat višja.

Pozimi 2012 so osebke *L. bocagei* vzorčili na petih mestih: Prado, Trofa, Ponte Junqueira, Graça in Caldas Vizela. Aktivnost GST je bila najvišja na mestu Ponte Junqueira (37,86 ± 5,40 nmol/min/mg protein), ki so mu nato sledila mesta Trofa, Graça in Prado. Najnižja vrednost aktivnosti GST je bila izmerjena na mestu s slabim ekološkim statusom Caldas Vizela (20,12 ± 4,70 nmol/min/mg protein).

GST spada med encime, ki katalizirajo konjugacijo GSH s spojinami, ki imajo reaktivno elektrofilno skupino (kovine, pesticidi) (Carvalho in sod., 2012). V diplomski nalogi so

bile višje vrednosti aktivnosti GST na onesnaženih mestih. Višje vrednosti GST na onesnaženih mestih so bile ugotovljene tudi v študiji s *Carassius gibelio* (Bloch, 1782), kjer so bile vrednosti v vzorcih iz reke Dnjeper višje kot vrednosti v kontrolnih vzorcev (Tsangaris in sod., 2011).

V vzorcih iz osebkov *Pseudochondrostoma* sp. je bilo opaziti sezonsko variacijo, saj so bile aktivnosti GST višje poleti. Sezonska variacija je bila ugotovljena tudi v študiji, ki so jo opravili Amado in sod. (2006), kjer so bile vrednosti izmerjene v osebkih *Micropogonias furnieri* (Desmarest,1823) višje v toplejših letnih časih.

Aktivnost GST v osebkih *L. bocagei* je bila poleti višja le na onesnaženem mestu Caldas Vizela (20,12 ± 4,70 nmol/min/mg protein). Rezultati morda ne odražajo realnega stanja, ali pa je prišlo do spremembe ekološkega statusa. Manjše vrednosti so lahko posledica poškodbe tkiv, saj so škrge v neposrednem stiku z onesnaževali (Dautremepuits in sod., 2009).

Pri obeh vrstah so bile vrednosti aktivnosti GST višje na onesnaženih mestih. Visoke vrednosti aktivnosti GST nakazujejo na prisotnost organskih onesnaževal (Ahmad in sod., 2005). Višje vrednosti aktivnosti GST lahko povzročijo tudi poliaromatski ogljivodiki (PAH) in pesticidi (Monteiro in sod. 2009).

Superoksid dismutaza (SOD)

Rezultati aktivnosti SOD so bili na voljo le za zimske vzorce (2012) iz osebkov *L. bocagei*. Pozimi 2012 so bili osebki *L. bocagei* vzorčeni na petih mestih: Prado, Trofa, Ponte Junqueira, Graça in Caldas Vizela. Med vzorčnimi mesti ni bilo statistične razlike ($p>0,05$); najvišje vrednosti aktivnosti SOD so bile izmerjene na mestih Prado (1,87 ± 0,37 U SOD/mg protein) in Graça (1,81 ± 0,35 U SOD/mg protein), ki sta jima sledila Ponte Junqueira and Caldas Vizela. Najnižje vrednosti so bile določene na mestu Trofa (0,96 ± 0,24 U SOD/mg protein). SOD je eden od prvih encimov v boju proti oksidativnem stresu, ki jih povzroča prisotnost kovin. Aktivnost SOD v osebkih *L. bocagei* je bila nizka. Ena izmed možnih razlag je inhibicija SOD zaradi vodikovega peroksida (H_2O_2) ali hidroksilnega radikala ($OH^{-\bullet}$), saj SOD katalizira razpad superoksidnih anionskih radikalov (Pandey et al., 2008).

Oksidativni stres v ribah in antioksidativni potencial se spreminja glede na vrsto uporabljene ribe, habitat in prehranjevalne navade (Ahmad in sod. 2004). V literaturi so opisane sezonske spremembe v oksidativnem stresu, ki so posledica abiotskih faktorjev kot so temperatura in vsebnost kisika. Nizka koncentracija kisika v vodi lahko vpliva na populacijo rib in povzroči smrt ali nenormalnosti pri potomcih. Temperatura in pH pa vplivata na katalitično učinkovitost in vezavno sposobnost encimov (Carvalho in sod., 2012).

Tako *Pseudochondrostoma sp*. kot tudi *L. bocagei* sta vrsti, ki se prehranjujeta na dnu rek, kar pomeni, da so lahko višje vrednosti aktivnosti encimov oksidativnega stresa posledica zaužitih onesnaževal v sedimentih rek.

Primerjava antioksidativnega odgovora v osebkih *Pseudochondrostoma sp.* in *L. bocagei* je možna le za LPO in aktivnost GST. Na mestu Caldas Vizela smo imeli vzorce obeh vrst tako poleti kot tudi pozimi. Vrednosti LPO so bile v osebkih *Pseudochondrostoma* sp. podobne poleti in pozimi. Poleti so lahko višje vrednosti LPO v osebkih *L. bocagei* posledica razmnoževalnega obdobja, ki traja od aprila do junija. LPO se ni izkazala kot dober indikator onesnaženosti. Podobno so ugotovili tudi v

študiji Pandey in sodelavci (2003). Avtorji navajajo, da je možen razlog za takšne rezultate maksimalna stopnja lipidne peroksidacije zaradi stalne izpostavljenosti rib onesnaževalom.

Rezultati aktivnosti GST v osebkih *Pseudochondrostoma* sp. se lahko primerjajo z referenčnimi mesti. Tako poleti kot pozimi so bile vrednosti GST višje na onesnaženih mestih (Caldas Vizela). Primerjava GST z referenčnimi mesti v osebkih *L. bocagei* ni bila možna, saj se ta mesta nahajajo višje v porečjih rek Ave in Douro, *L. bocagei* pa ne naseljuje zgornjih tokov. Najbolj onesnaženo mesto glede na dobljene rezultate je Caldas Vizela, kar je v skladu s klasifikacijo ekološkega stanja glede na Vodno direktivo.

Največja ovira pri interpretaciji rezultatov je bilo majhno število vzorcev. Za poglobljen monitoring je potrebno analizirati večje število živali skupaj s fizikalnim in kemijskim monitoringom.

ZAKLJUČEK

Nizko aktivnost LPO smo ugotovili v osebkih *Pseudochondrostoma* sp. kot tudi pri *L. bocagei*. LPO je bila višja poleti v primerjavi z zimskim letnim časom. Na višje vrednosti LPO lahko vpliva več dejavnikov: višja temperatura, ki lahko poveča stopnjo dihanja v mitohondrijih čemur sledi povečana produkcija ROS (Oliva in sod., 2012). Višja temperatura pomeni manj raztopljenega kisika, kar je lahko razlog za stres ali celo smrt v ribjih populacijah (Carvalho in sod., 2012).

Aktivnost GST je bila v osebkih *Pseudochondrostoma* sp. višja v poletnem času. Nižje vrednosti so bile izmerjene na mestih z dobrim ekološkim stanjem (Tejão and Pinhão). Pri *L. bocagei* pa je bila situacija obrnjena. V *L. bocagei* so bile aktivnosti GST višje pozimi 2012. Vzorčno mesto Caldas Vizela je imelo najvišje vrednosti GST pri obeh vrstah v poletnem času. Visoke vsebnosti GST nakazujejo prisotnost organskih onesnaževal (Ahmad in sod., 2006), najverjetneje zaradi kmetijskih in industrijskih izpustov poleti.

Aktivnost SOD je bila določena le v *L. bocagei* pozimi 2012. Aktivnosti SOD so bile nizke in ni bilo vzorcev iz vzorčnih mest z dobrim ekološkim stanjem, ki bi lahko služili za primerjavo.

Predvidevali smo, da bodo imela mesta s slabim ekološkim statusom višje vrednosti encimskih aktivnosti zaradi prisotnosti onesnaževal. To je bilo potrjeno z izmerjeno aktivnostjo GST. Višje izmerjene aktivnosti GST so lahko indikator prisotnosti organskih onesnaževal na mestih s slabim ekološkim statusom. Za bolj natančno in pravilno interpretacijo rezultatov bi bilo potrebno ugotoviti povezavo med parametri kvalitete vode (fizikalne in kemijske meritve) in biološkimi odzivi na vzorčnem mestu. To je potrebno, ker na fiziološke procese rib vpliva več faktorjev (razmere v habitatu, razpoložljiva hranila, fizikalni in kemijski parametri kot so temperatura in raztopljen kisik) (Pereira in sod., 2013).

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

ABBREVATIONS

BHT = Butylated hydroxytoluene
BSA = Bovine serume albumin
CAT = Catalase
CDNB = 1-chloro-2,3-dinitrobenzene
DNA = Deoxyribonucleic acid
EPA = Environmental Protection Agency
EROD = Ethoxyresorufin-O-deethylase
GPX = Glutathione peroxidase
GSH = Glutathione
GST = Glutathione-S-transferase
LPO = Lipid peroxidation
MDA = Malondialdehyde
MS-222 = Tricaine methanesulfonate
NRC = National Research Council
NPS = Non-point source pollution
PUFAs = Polyunsaturated fatty acids
ROS = Reactive oxygen species
SOD = Superoxide dismutase
TBA = Thiobarbituric acid
TBARS = Thiobarbituric acid reactive substances
TCA = Trichloroacetic acid
WWTP = Waste Water Treatment Plant

1 INTRODUCTION

In the last century, we have witnessed worldwide industrial development which has led to the pollution of the air, water and soil. With the growth of human activities an increasing number of chemicals are continuously being released in the environment due to industrial activities, and a lot of these chemicals represent a serious threat to living organisms and ecosystems (Henzová et al., 2008).

The increase in production and application of pesticides in agriculture as well as for plant protection and animal health has resulted in the contamination of soil, ground and surface waters. This creates a hazard to the environment and also to human health, either through direct exposure or through residues in food and drinking water. Alarming levels of pesticides have been reported from some places in air, water, soil as well as in food and biological materials. Some of these compounds are not easily degradable and they are also toxic, mutagenic and carcinogenic (Aksu, 2005).

Aquatic ecosystems are, like other ecosystems, subject to toxic contaminants coming from industrial, agricultural and domestic activities (Oliveira et al., 2010). One of the biggest problems is the release of heavy metals in freshwater and marine ecosystems from different origins such as industrial wastewaters, petrochemical, refining, fertilizer, steel and car industries (Henzová et al., 2008). Likewise, pesticide residues reach the aquatic environment through manufacturing plants, direct surface run-off, leaching, careless disposal of empty containers and equipment washing (Aksu, 2005).

For the improvement of the quality of ecosystems it is necessary to know which water bodies are impaired and which factors cause environmental deterioration. Physical, chemical and bacteriological measurements are commonly the basis of water monitoring, as they provide a wide spectrum for proper water management (Metcalfe, 1989).

Even though chemical pollution plays a primary role, only chemical analyses through periodic measurements of chemicals (environmental monitoring) may not be sufficient in describing the adverse effects of the complex mixtures of chemicals present in contaminated sites (Ozmen et al., 2008). This is especially evident in running waters, where changes in hydrology are rapid, and therefore difficult to estimate, and cannot reflect the integration of numerous environmental factors and the long-term sustainability of river ecosystems due to their instantaneous nature (Li et al., 2010).

The concentrations of substances in surface water are normally low, but usually pollutants are present for long periods of time. Under these conditions, these substances can act chronically. Therefore, the impact of these compounds usually does not manifest itself directly by death of affected organisms, but more commonly by failure of important vital functions which decrease the organism's vitality (Soldán, 2003).

As a result, biomonitoring has been proven to be a necessary and efficient supplement to the traditional monitoring techniques (Soininen & Könönen, 2004). Biological monitoring uses living organisms for the determination of environmental pollution, due to the fact that pollution may elicit both adaptive and adverse responses in animals at different structural levels (cells, tissues, and organs). These reactions are complex and depend on a variety of factors, such as the type of contaminant and its concentration, the rate of exposure, and the susceptibility of each organ. Another relevant factor to the

final toxic effect is the synergistic or antagonistic interaction between pollutants (Fenoglio et al., 2005).

Normally, the responses to pollution are reflected as changes in some enzyme activities, especially key enzymes of biotransformation systems of organisms which can be used as biomarkers and provide a specific tool for pollution effects evaluation (Ozmen et al., 2008). Fish species are the most commonly used vertebrate model in ecotoxicological studies (Fernandes et al., 2008) due to their sensitivity to pollution, special biological characteristics, for instance their relatively big body size and long life cycle. Furthermore, fish species are at the top position in the aquatic food chain, which means that there is a possibility of a direct effect on human health (Zhou et al., 2008).

1.1 Work aims:

- Evaluation of pollution effects at sampling sites with different ecological status using biomarkers of exposure;
- evaluation of biomarkers of exposure (oxidative stress and cell damage) in fish species *Pseudochondrostoma* sp. and *Luciobarbus bocagei*:
  - estimation of lipid peroxidation;
  - activity of glutathione-*S*-tranferase;
  - activity of superoxide dismutase;
- comparison of results between species, among different sampling sites and among seasons (winter and summer).

2 THEORETICAL PART

2.1 Aquatic environment and pollution

According to the United Nations Convention on the Law of Sea (1982), pollution has been defined as "*the introduction by man, directly or indirectly, of substances or energy into the marine environment, including estuaries, which results or it is likely to result in such deleterious effects as harm to living resources and marine life, hazards to human health, hindrance to marine activities, including fishing and other legitimate uses of the sea, impairment of quality of use of the sea water and reduction is amenities*".

The environment is continuously being polluted and most often the ultimate sink for disposing pollutants is the aquatic environment. This may be due to direct discharges or as a result of hydrological and atmospheric processes (Stegeman and Hahn, 1994; van der Oost et al 2003). Sources of pollution can be divided into point and nonpoint sources. The term "source point" according to the Clean Water Act (1972) (section 502(14)) means "*any discernible, confined and discrete conveyance, including but not limited to any pipe, ditch, channel, conduit, well, discrete fissure, container, rolling stock, concentrated animal feeding operation, or vessel or other floating craft, from which pollutants are or may be discharged and does not include agricultural storm water discharges and return flows from irrigated agriculture"*. NPS pollution is a pollution that comes from many diffuse sources. Pollution runoff is caused by rainfall or snowmelt moving over and also through the ground. Moving runoff carries away natural and anthropogenic pollutants, depositing them into watersheds through lakes, rivers, wetlands and coastal waters (EPA, 2005). Nonpoint source pollutants can among others include excess fertilizers, herbicides and insecticides from agricultural lands and residential areas, oil, grease and toxic chemicals from urban runoff and energy production (EPA, 2012).

Production and emissions of contaminants are usually derived from human settlements, development and construction, resource uses and interventions, agricultural activities, industrial development, urbanization and tourism (Islam and Tanaka, 2004). Much of the waste from these activities contains a wide variety of organic and inorganic pollutants including solvents, oils, grease, plastics, plasticizers, phenols, heavy metals and suspended solids, as well pesticides, herbicides, salts and other potentially hazardous substances (Pandey et al., 2003; Parvez et al., 2006). Other contaminants of major concern include radionuclides, pathogens, litter, sediments and debris (Williams, 1996).

2.1.1 Coastal areas

Most of the coastal areas of the world have been reported to be to some degree polluted, particularly commercial coastal and marine fisheries have proven significantly affected (Islam and Tanaka, 2004). Globally, more than three billion people live in the proximity to the marine coast, meaning that wastes from industrial and domestic sources as well as habitat destruction have a significant impact on coastal environments (Rice, 2003; Torres et al., 2008).

Other surface waters such as estuaries, rivers and lakes also receive large quantities of waste water (Ohe et al., 2004) from anthropogenic activities – the main factor

leading to the increasing levels of contaminants in all aquatic environments (Cazenave et al., 2009).

2.1.2 Estuaries

Estuaries, coastal environments that are the interface between land and sea, have drawn scientific interest, since they are a complex mixture of salt and fresh water. They are productive natural habitats where large phytoplankton populations support a variety of other organisms that include a lot of commercially important marine fish and crustacean species that use them as nursery grounds (Cattrijsse et al., 1994).

In Portugal, the most important metropolitan areas grew around the estuaries of river Tejo and Douro. These areas have also very important harbour activities (Cardoso da Silva and Carmona Rodrigues, 2004). A good example from Portugal is Ria de Aveiro. It has been the main receptor of anthropogenic discharges resulting mainly from chlor-alkali and pulp/paper plants, harbor and dry-dock activities as well as municipal and domestic effluents. This ecosystem, like other estuaries, is a nursery area for juvenile stages of several fish species including the European sea bass (*Dicentrarchus labrax, Linnaeus, 1758*), which has an important ecological role and displays a high commercial value, as it is the basis of professional and recreational fishing activities (Ahmad et al., 2008).

2.1.3 Rivers and lakes

Since some rivers and lakes are used as water supplies as well as for agricultural, recreational and religious activities all around the world (Ohe et al., 2004), their pollution is not only a threat to the environment, but also to human health. In aquatic systems, a lot of attention has been drawn to the wide occurrence of metal pollution. Some of the heavy metals have the ability to transform into persistent metallic compounds that have the ability to bioaccumulate in organs and can be magnified along the food chain.

In a research conducted by Naigaga et al. (2011), the environmental water quality of Lake Victoria wetlands (Uganda) was investigated, as Lake Victoria wetlands are being extensively degraded due to human activities, such as habitat modification for agriculture, municipal and animal waste disposal and urbanization. The results showed that the distribution and abundance of fish species was strongly influenced by water quality. The study showed that sites with higher nutrient values, especially a total phosphorous concentration, registered low fish biodiversity and richness, indicating that eutrophication is connected with reduced fish biodiversity. Furthermore, the observed decline of fish species and fish stocks in Lake Victoria itself may have been caused by overexploitation, use of destructive fishing gear and methods, environmental degradation, and ineffective or absent management of the lake.

Investigations on the Yangtze River showed the occurrence of various levels of heavy metals along shore-aquatic areas with the predominant elements of zinc (Zn), lead (Pb), cadmium (Cd) and chromium (Cr) (Zhou et al., 2008). Furthermore, there are other families of unregulated compounds known as “emerging substances”, namely chemicals from consumer products, pharmaceuticals or other chemical of agricultural and industrial activities whose effects still have to be seen and can alter both the chemical and ecological quality of water (Gomez et al., 2012). These pollutants also possess the potential to cause oxidative stress through free radical and reactive oxygen species mechanisms.

Rivers in Portugal are prone to pollution due to human activities. Ave River is 98 km long and is situated in Northern Portugal. The drainage basin covers approximately 1388 km^2 (Gonçalves et al., 1992). The most important tributaries are the Este River (51 km length, 245 km^2 drainage area) and the Vizela River (47 km, 240 km^2). Over the last three decades, the main watercourse of this river has been subjected to multiple discharges of untreated wastewater related to the high industrial density in the middle and lower parts of the river basin. The most influential is the textile sector, because it includes more than 70% of all people employed in industry in this area. Apart from that, leather tanning, rubber manufacture and plastic production also played a significant role in polluting Ave River (Soares et al., 1999). As a result of discharging untreated water, the quality of the river has deteriorated and has resulted in water that is not only inappropriate as a water supply for domestic and industrial use, recreational purposes, fishery and irrigation, but it also poses danger to flora, fauna and public health. The main surface water quality problems include organic pollution, turbidity and colour problems, eutrophication, specific pollutants due to agriculture, heavy metals, natural organic matter and organic metal compounds (Oliveira et al., 2005).

Another important river in Portugal is Douro River which is one of the longest rivers of the Iberian Peninsula with the length of 930 km. In Portugal, this river surface and underground resources are used for the production of electricity (53% of the national total), irrigation, industrial purposes and also as a water supply for half of the population living in the metropolitan area of Porto (Cortes, 2009; Madureira et al., 2010). The biggest anthropogenic stress on the Douro River results from industrial development and WWTPs effluents that are discharged into the estuary or its tributaries. The Douro River estuary receives, directly or indirectly, effluents from eight WWTPs, two of the largest (Sobreiras and Freixo) being located in the lower and medium stretch of the estuary. Areas of extreme pressure are the first nine km of the mouth of Douro River estuary due to the densely populated area, since two major cities, Porto and Vila Nova de Gaia, are located on the north and south banks (Madureira et al., 2010).

2.2 Monitoring of aquatic pollution

Since the aquatic environment is constantly subjected to toxic contaminants resultant from human activities, the monitoring of these contaminants in water, sediments and biota by itself is of crucial importance. Even though the chemical analysis of the environment is the most direct approach to reveal the status of the environment, it has its limitations. The main disadvantage is that it cannot afford the powerful evidence on the integrated influence and possible toxicity of such pollution on organisms and ecosystems. Therefore, biomonitoring as a scientific technique for assessing the environment including human exposure to natural and synthetic chemicals is essential (Zhou et al., 2008). The term biomonitoring or biological monitoring can be defined as a regular, systematic use of living organisms in order to evaluate changes in environmental or water quality (de Zwaart, 1995), based on sampling and analysis of an individual's tissues and/or fluids (Zhou et al., 2008). This takes the advantage of the knowledge that chemicals which enter the organisms leave marks reflecting the exposure (Zhou et al., 2008).

Environmental toxicity studies on aquatic organisms have focused primarily on redox cycling compounds such as quinones, aromatic hydrocarbon-quinones, nitropyrene, lindane, paraquat and nitrobenzoic acid (Valavanidis et al., 2006). Since the ultimate sink for many of these contaminants is the aquatic environment (Stegeman and Hahn,

1994), the indiscriminate dumping and release of wastes of the above mentioned hazardous substances into rivers might lead to environmental disturbance which could be a potential source of stress to the biotic community (Donaldson, 1981; op. cit.: Pandey et al., 2003).

Aquatic organisms can uptake pollutants via sediments, suspended particulate matter and food sources. Also, exposure to contaminants will depend on the dietary and ecological lifestyles of each organism (Valavanidis et al., 2006). Enzymatic antioxidants ubiquitous in animal species and different tissue types are found widely in aquatic organisms and measurements of their depletion/increase can be used as biomarkers for assessing the effects of xenobiotics (Livingstone et al., 1994). Aquatic organisms are more sensitive to toxicity and exposure when compared to terrestrial organisms. Therefore, they may provide experimental data for the evaluation of subtle effects of oxidative stress, mutagenic effects and other adverse effects of pollutants (Lackner, 1998). Over the years, marine invertebrates, especially bivalve mollusks (i.e. mussels and oysters), have been extensively chosen as test species due to their filtration capability, ease of caging and sensitivity to oxidative damage from concerning chronic exposure or sublethal concentrations (Valavanidis et al., 2006).

2.3 Aquatic bioindicators

Among different species, considerable variation in both the basic physiological features and the responsiveness of certain biomarkers towards environmental pollution may become apparent, but despite their limitations, such as relatively high mobility, fish are generally considered to be the most feasible organisms for pollution monitoring in aquatic systems (van der Oost et al., 2003).

2.3.1 Fish as bioindicators

Fish have attracted much attention in the biomonitoring of water pollution due to their special biological characteristics which include a relatively large body size and a long life-cycle. Furthermore, fish are at the top of the aquatic food chain, which is significant for biomonitoring purposes (Zhou et al., 2008). Numerous fish species meet the criteria for the use as bioindicators (Table 1) (van der Oost et al., 2003).

Table 1: *Characteristics of good bioindicators (Holt & Miller, 2002: pp. 8)*

Good indicator ability	Provide measurable response (sensitive to the disturbance or stress but does not experience mortality or accumulate pollutants directly from their environment)
	Response reflects the whole population/community/ecosystem response
	Respond in proportion to the degree of contamination or degradation
Abundant and common	Adequate local population density (rare species are not optimal)
	Common, including distribution within area of question
	Relatively stable despite moderate climatic and environmental variability
Well-studied	Ecology and life history well understood
	Taxonomically well documented and stable
	Easy and inexpensive to survey
Economically/commercially important	Species already being harvested for other purposes
	Public interest in or awareness of the species

In the research conducted by Pandey and collaborators (2003), Indian freshwater fish *Wallago attu* (Bloch & Schneider, 1801) was used as a species for determining various biomarkers of oxidative stress. Furthermore, in a study done by Tsangaris and collaborators (2011), Prussian carp (*Carassius gibelio,* Bloch, 1782) was used for toxicity testing in Ukrainian polluted river waters. There are numerous studies that used different fish species for assessing oxidative stress e.g. carp (*Cyprinus carpio,* Linnaeus, 1758) (Hao and Chen, 2012), *Geophagus brasiliensis* (Quoy & Gaimard, 1824) (Linde-Arias et al., 2008), *Channa punctata,* (Bloch, 1793) (Parvez and Raisuddin, 2005).

As indicators of exposure to pollution, contaminant levels in fish may be used. However, it should be noticed that factors such as age, sex, season and mobility may play an important role. It is hard to detect pollutants in fish blood, which means that in order to assess the level of pollution different fish organs have been used, particularly gills, liver, kidney, muscle or the whole body (van der Oost et al., 2003).

2.3.1.1 Gills

Gills play a central role in the physiological balance of fish, which is based on a variety of functions, such as gas exchange, osmotic and ionic regulation, acid-base balance, and nitrogenous waste (Ahmad et al., 2008; Monteiro et al., 2009). Fish gills are sensitive respiratory and ion-regulating membranes and are the first point of contact

between waterborne contaminants and fish. In general, low concentrations of waterborne metals have ion-regulating effects in gills, whereas higher concentrations cause gill damage and mucus accumulation (Playle, 1998).

The gills of most teleosts are usually composed of four pairs of gill arches, supported by a bone skeleton. Filaments come from the gill arches that are supported by cartilage, from which secondary lamellae exit. The secondary lamellae are constituted by a simple epithelium, where gas exchange occurs (Marques dos Santos et al., 2011). The gill epithelium is found lining the arch, trails, primary lamellae and interlamelar regions. This epithelium is stratified and consists of different cell types, including squamous cells, mucus-secreting cells and cells rich in mitochondria (Evans et al., 2005). The second epithelium type is the respiratory epithelium that covers the respiratory lamellae (Marques dos Santos et al., 2011).

The mucus-secreting cells can usually be found in the filaments, and mucus can be found on the respiratory epithelium in fish that are or have been exposed to stress conditions, indicating that the mucus layer protects the lamellae surface against infectious agents, toxicants and particulate matter (Powell et al., 1992; Marques dos Santos et al., 2011).

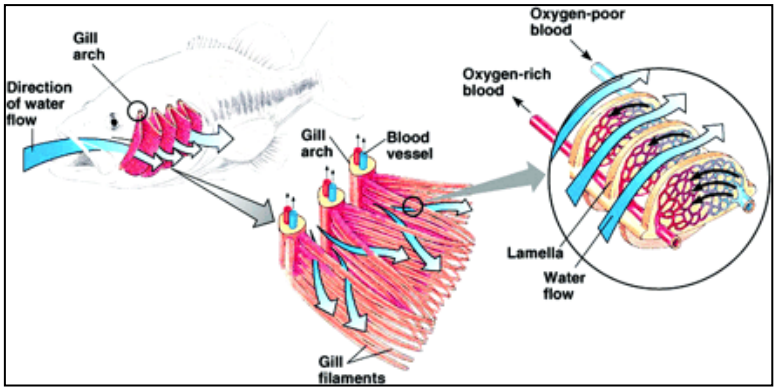

Figure 1: *Structure of the gill in the teleost fish and flow of water over gills (after Campbell and Reece, 2002; op. cit.: Evans et al., 2005).*

The multifunctionality of gills, the large surface area they occupy and their location, which is directly related to the external environment, make the gills a key organ for understanding the relation of fish to the environment and the action of pollutants in the aquatic environment (Marques do Santos et al., 2011).

2.4 Oxidative stress

More than fifty years ago, it was discovered that free radicals are present in biological systems and immediately they were linked to diseases and aging (Harman, 1956). Oxygen and nitrogen free radicals are of utter importance in the physiological control of cell function. There are numerous physiological functions that involve free radicals or their derivates, among others being sensing of oxygen tension, regulation of vascular tone and enhancement of signal transduction from various membrane receptors (Dröge, 2002). Basic metabolism in aerobic organisms involves the production of oxygen free radicals and non-radical reactive species, otherwise known as ROS (Valavanidis et al., 2006).

Free radicals are atoms, ions or molecules which have unpaired electrons that are usually very reactive. As a consequence of being reactive, they are likely to take part in chemical reactions (Lushchak, 2011). Even though free radicals possess injuring potential, which is realized in living organisms, their level is under strict control in order to prevent damages. This balance is very delicate and it may be disturbed, which can lead to perturbations in redox status (Lushchak, 2011). Free radicals and theirs derivatives often regulate many different processes such as hormonal response in plants (Apel and Hirt, 2004) and in animals (Winterbourn and Hampton, 2008).

The hydroxyl radical ($HO^{\bullet}$) with the life span of a few nanoseconds is the most important free radical with a very high toxicological and biological importance due to its high reactive potential and indiscriminate reactivity with cellular components, such as lipids of biological membranes, DNA and proteins of enzymes (Levine et al., 2000).

ROS are continuously produced in biological systems either as side products of aerobic metabolism or products of specialized systems designed to produce ROS. They can be bound or detoxified by different types of antioxidants or can interact with cellular or extracellular components. The metabolism of ROS is under strict cellular control due to their high capacity of damage and biological activity (Luschak, 2011). As ROS are continuously generated and eliminated, usually the amount of ROS is virtually equal to the eliminated one. The ROS concentration is a dynamic parameter therefore it is necessary to talk about steady-state ROS concentration (Lushchak, 2011). It is possible that during metabolic processes a small portion of free radicals may escape from the shield of antioxidant mechanisms, resulting in oxidative damage of cellular components (Valavanidis et al., 2006).

During evolution biological systems have developed a defence against oxidative damage using adequate enzymatic and non-enzymatic antioxidant mechanisms. Enzymatic antioxidants connected with oxidative stress are SOD, CAT and GPX (de Zwart et al., 1999). The non-enzymatic antioxidant systems are mostly substances of low molecular weight, such as vitamins C and E, urate, retinyl esters, ß-carotene and GSH (Kohen and Nyska, 2002). GSH may serve as a cofactor for antioxidant enzymes such as on glutathione-dependent peroxidases, or GSTs, which are a second phase detoxification enzyme (Lushchak, 2011). The imbalance between the generation and elimination of ROS by such mechanisms is termed oxidative stress (Davies, 1995).

Oxidative stress is a situation when steady-state ROS concentration is transiently or chronically enhanced, disturbing cellular metabolism and its regulation and damaging cellular constituents. Reductive stress may be defined in a similar way with the difference that steady-state ROS concentration decreases (Lushchak, 2011).

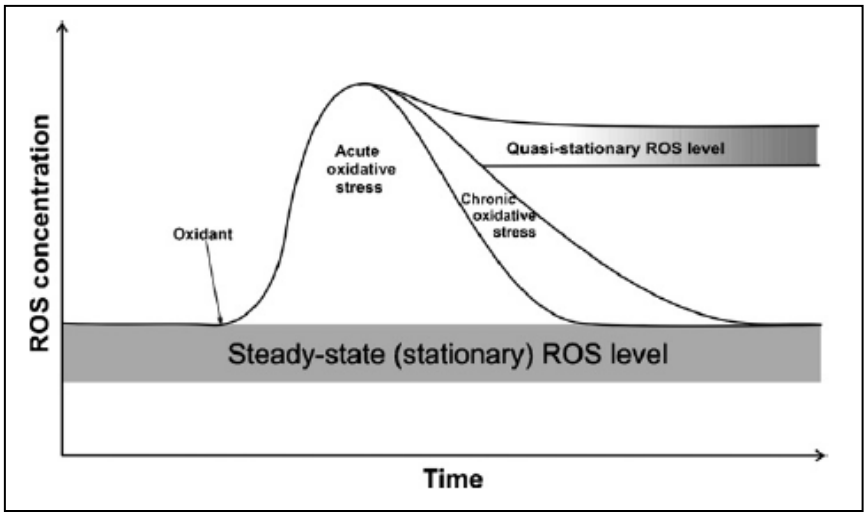

Figure 2: *Schematic presentation of oxidative stress: The dynamics of oxidant-induced perturbation of level or reactive oxygen species (after Lushchak, 2011).*

If the conditions are normal, the ROS level is stabilized at a certain level provided by the balance between ROS production and elimination. Oxidative stress results in the enhancement of the ROS steady-state level and if the antioxidant potential is high enough, the ROS level will return into its initial state. A transient increase of the ROS level is termed “acute oxidative stress”. If the efficiency of the antioxidant system does not counterbalance enhanced ROS production quickly, the system may need to recover from the stress longer, which is termed “chronic oxidative stress”. Nevertheless, the system may be stabilized at a higher ROS level than at the beginning – “quasi-stationary ROS level” (Lushchak, 2011).

There are several mechanisms of ROS generation in biological systems. They are mostly produced as side-products of oxygen metabolism. More than 90% of oxygen that is consumed is used via four-electron mechanisms by electron-transport chains related to the production of energy (Papa and Skulachev, 1997).

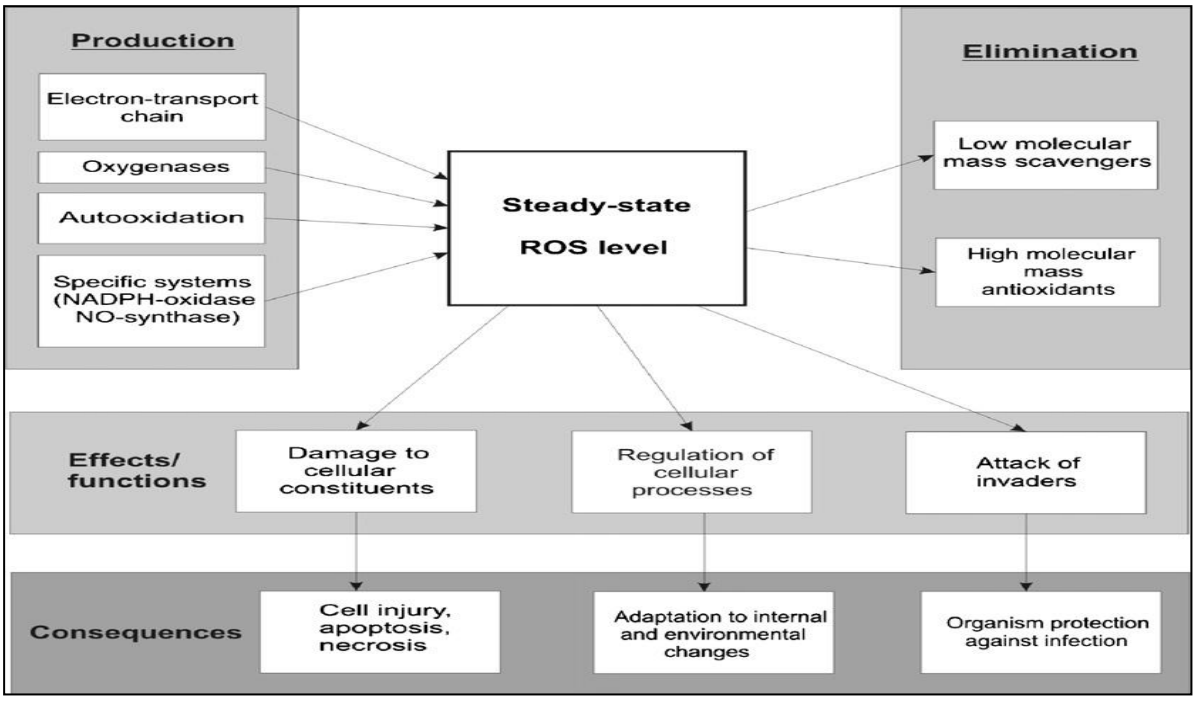

Figure 3: *Balance between production and elimination of ROS and their potential biological effects (after Lushchak, 2011).*

Important sources of ROS in biological systems are chemical toxic pollutants, namely xenobiotics that are capable of redox cycling. Molecules that are known for their redox cycling properties and oxidative stress potential include transition metals, quinines, dyes, biprydyl herbicides, and aromatic nitro compounds (Kappus and Sies, 1981).

The steady-state ROS level is provided with a balance between generation and elimination of ROS. If some portion of ROS escapes defence systems and affects biological systems, this will lead to the damage of cellular components, induction and modification of regulatory cascades and to the attack of invaders. The latter three events can then lead to cellular injury through different mechanisms, adaptation to new conditions and also the protection of the host against infections (Lushchak, 2011).

2.5 Biomarkers

To test oxidative damage in biomolecules and various aspects of oxidative stress due to free radicals in experimental animals, biomarkers are used. Biomarkers can, in addition to using primary and secondary products of free radicals damage, also monitor the status of various antioxidant defence mechanisms against free radicals (Lushchak, 2011).

Biomarkers are measured in body fluids, cells or tissues indicating biocellular or biochemical alterations as a result of the presence and magnitude of toxic substances, or of the host response (NRC, 1987). Good biomarkers are sensitive indicators of both pollutant bioavailability and early biological responses and can be used after the exposure to a dietary, environmental or occupational source in order to determine the connection between cause-effect and dose-effect relationship for the purpose of health

risk assessment in clinical studies and for monitoring purposes (Suter, 1990; van der Oost et al., 2003).

The use of biomarkers for monitoring environmental pollution has gained considerable interest for the purposes of assessing river conditions across the globe (Pandey et al., 2003; Cazenave et al., 2009), since they can complement and enhance the reliability of chemical analysis data, resulting in more integral and biologically relevant information regarding the potential impact of toxicants on the health of organisms (van der Oost et al., 2003; Parvez et al., 2006; Cazenave et al., 2009).

Fish biomarkers may be useful tools in several steps of the risk assessment process: effect, exposure, and hazard assessment, risk characterization or classification, and monitoring the environmental quality of aquatic ecosystems (van der Oost et al., 2003).

2.5.1 Lipid peroxidation (LPO)

Lipid peroxidation is the oxidation of PUFAs, which are very sensitive to oxidative reactions by ROS due to their double bonds. It is an important consequence of oxidative stress (van der Oost, 2003) and LPO products represent a constant threat to the aerobic cells (Valavanidis et al., 2006). The process is composed of a set of chain reactions and is initiated mainly by hydroxyl radicals, especially in transition metal-catalyzed reactions (Valavanidis et al., 2006). Biomarkers of LPO are probably the most extensively investigated process in tissue injury caused by free radicals, however as direct analysis of endogenous LPO is difficult, most methods measure levels of secondary oxidation products (Valavanidis et al., 2006).

2.5.2 Superoxide dismutase (SOD)

SODs belong to a group of metalloenzymes that are responsible for catalyzing the conversion of reactive superoxide anions ($O_2^{-\bullet}$) in order to generate hydrogen peroxide (H_2O_2), which is an important ROS (van der Oost et al., 2003). They are considered to play a crucial antioxidant role as their importance is indicated in all aerobic organisms examined (Stegeman et al., 1992; op cit: van der Oost et al., 2003). Most techniques for the determination of the SOD activity are indirect assays in which an indicating scavenger competes with endogenous SOD for $O_2^{-\bullet}$. A unit of SOD activity is defined as the amount that causes 50% inhibition of the reduction of the scavenger under specific conditions (Sazuka et al., 1989; van der Oost et al., 2003).

2.5.3 Glutathione *S*-transferase (GST)

GSTs (EC 2.5.1.18) are isoenzymes that catalyze the conjugation of electrophilic compounds to GSH. GSTs exist as dimeric proteins that are comprised of two units. Each of the subunits consists of a glutathione-binding site and a site for the binding of the electrophilic substrate (Chubben et al., 2001). On the basis of the substrate specificity, immunological cross-reactivity and protein sequence data, the soluble GSTs can be classified into four groups: a, m, p and q (George, 1994; op. cit.: van der Oost et al., 2003). Apart from their vital function in the intracellular transport systems (heme, bilirubin and bile acids) (van der Oost et al., 2003), they also participate in the defence against oxidative stress since these enzymes have the capability to detoxify endogenous harmful molecules such as hydroxylalkenals and base propenals or DNA hydroperoxides. In addition, they can also detoxify electrophilic xenobiotics and/or reactive intermediates that are formed during their biotransformation (Tew, 1994;

Chubben et al., 2001). Most studies use artificial substrate CDNB for the determination of the total GST activity. CDNB is conjugated by all GST isoforms, with q-class enzymes being an exception (George, 1994; op. cit.: van der Oost et al., 2003).

3 MATERIALS AND METHODS

The work was carried out at the University of Trás-os-Montes and Alto Douro in Vila Real, Portugal at the Department of Biology and Environment. The detailed list of chemicals used for the experiment is in Appendixes A, B, C and D.

3.1 Instruments and equipment

- pH meter
- balance (Scaltec SBC 21), accuracy ±0.0001 g
- centrifuge (Sigma K3)
- homogeniser (Potter-Elvenhjem homogeniser)
- vortex (TechnoKartell TK3S)
- spectrophotometer (Biotek PowerWave XS2)
- 96 well plate

3.2 Sampling area

The sampling was carried out in the summer of 2011 and the winter of 2012 and fish of the genus *Pseudochondrostoma* (Robalo, Almada, Levy and Doadrio 2007; Cyprinidae: Leuciscinae) and species *Luciobarbus bocagei* (Steindachner, 1864) were collected at the sampling sites with different degrees of contamination in the Ave, Cávado and Douro rivers basins.

The sampling sites were selected based on a geographic distribution of particular species along the main channels, taking into account the various types and sources of contamination as well as the selection of unpolluted reference points. Unpolluted reference source were sites with good ecological status (see Table 2). The sampling sites were: Ponte de Pingue, Prado, Ponte da Junqueira, Ponte Trofa, Vizela Santo Adrião, Tejão, Pinhão, Graça, and Caldas de Vizela (Ponte Romana). The geographical coordinates of the sampling locations are listed in the Appendix E.

Table 2: *Ecological status of the sampling sites according to Water Framework Directive (WFD, 2000).*

Sampling Site	Ecological status
Ponte de Pingue	Good
Tejão	Good
Pinhão	Good
Ponte Junqueira	Poor
Prado	Poor
Trofa	Poor
Vizela Santo Adrião	Poor
Graça	Bad
Caldas de Vizela	Bad

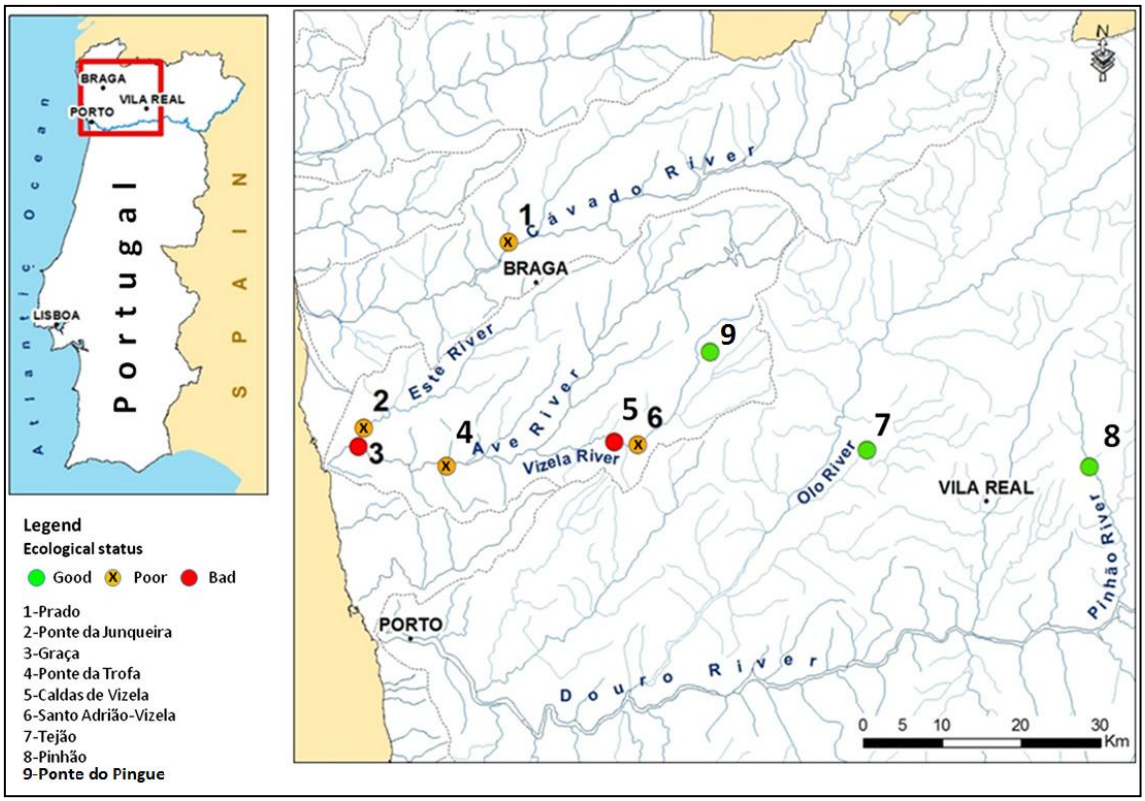

Figure 4: *Map showing the sampling locations with attributed ecological status (adapted from Pereira et al., 2013).*

3.3 Fish used as bioindicators

Pseudochondrostoma (Robalo, Almada, Levy and Doadrio 2007; Cyprinidae: Leuciscinae) is a genus endemic to the Iberian Peninsula with three species: *P. duriense* (Coelho, 1985), *P. polylepis* (Steindachner, 1864*) and P. wilkommii* (Steindachner, 1866). All species live in the middle stretches of rivers in areas with current. They are spread from Douro drainage to Galicia and feed on aquatic plants, small invertebrates and detritus. All three species spawn during spring. *P. duriense* and *P. polylepis* are found in northwestern parts of Portugal, meanwhile *P. willkommii* inhabites southeastern parts of the Iberian Peninsula (Kottelat and Freyhof, 2007). As it is difficult to distinguish between *P. duriense* and *P. polylepis,* caught specimens were determined to the genus level only. Morphologic data for *Pseudochondrostoma* sp. are presented in Appendixes F and G.

Figure 5: *Pseudochondrostoma duriense (left) (© Filipe Ribeiro)*[1] *and Pseudochondrostoma polylepis (right) (© A.C. Sostoa)*[2].

Luciobarbus bocagei (Steindachner, 1864; Cyprinidae: Barbinae), commonly known as the Iberian barbell, lives in middle and lower reaches of stream with slow current. *L. bocagei* is distributed along the Atlantic coast of Spain and Portugal from Lima to Sado drainages. Food is mainly benthic invertebrates and detritus and spawning time is late spring to early summer. The males reach sexual maturity at three years of age and females later at the age of eight years (Kottelat and Freyhof, 2007). Morphologic data for *L. bocagei* are presented in Appendixes H and I.

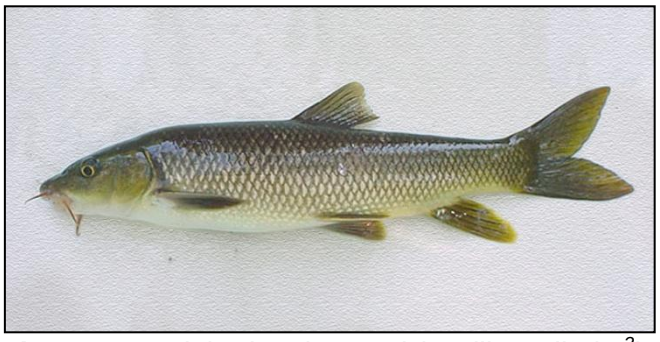

Figure 6: *Luciobarbus bocagei (© Filipe Ribeiro*[3]*).*

3.4 Preparation of tissue for enzymatic tests

Fish were captured using direct current backpack electrofishing equipment with a DC-500 V generator. Fish were anaesthetized by immersion in MS-222, measured and weighed. MS-222 is an anaesthesia agent used for anaesthesia and euthanasia of poikilothermic animals (Cakir & Strauch, 2005). They were euthanized by decapitation and gills samples were removed, frozen in liquid nitrogen and stored at -80°C until biochemical assay.

[1] Source: http://www.arkive.org/northern-straight-mouth-nase/pseudochondrostoma-duriense/factsheet 17.6.2013
[2] Source: http://www.ittiofauna.org/webmuseum/pesciossei/cypriniformes/cyprinidae/pseudochondrostoma/pseudochondrostoma_polylepis/index_big.htm 17.6.2013
[3] Source: http://www.ittiofauna.org/webmuseum/pesciossei/cypriniformes/cyprinidae/luciobarbus/luciobarbus_bocagei/l_bocagei.htm 17.6.2013

Gills were homogenised in homogenization buffer (100 mM potassium phosphate buffer (100 mM KH_2PO_4/K_2HPO_4) and 1 mM Na_2EDTA, pH=7.5). The gill samples were defrosted, weighted and put into test tubes with tweezers. I annotated the weight and put gill samples in a Potter tube and added homogenization buffer (1 mL of homogenization buffer for 100 mg of gill sample) and homogenized with Potter-Elvenhjem homogeniser for 1 to 2 minutes with the tube placed on ice. After homogenization the samples were transferred into 1.5 mL eppendorf tubes. After every sample the Potter tube and the plunger was washed with distilled water.

After homogenizing the samples, tissue homogenates were centrifuged at 15 000 rpm for 20 minutes at 4°C. The supernatant was collected and transferred into new 1.5 mL eppendorf tube. An aliquot of 200 µL of the supernatant was transferred into another 1.5 mL eppendorf tube for determination of lipid peroxidation and an aliquot of 25 µL of supernatant was transferred into eppendorf tube for protein determination. The remained supernatant was collected and used for determination of enzymes activities (GST, SOD). All the manipulations were done on ice and the samples were stored at -80°C.

3.5 Lipid peroxidation (TBARS Assay)

Lipid peroxidation assay was carried out according to the Niki (2000). 25 mL of solution A was prepared (consisted of 1 % TBA, 0.05 M NaOH and 0.025 % BHT). TBA was added to 1 M NaOH and H_2O and warmed until TBA dissolved. BHT was prepared in 1 mL ethanol and added to the previous solution. H_2O was added up to 25 mL. Immediately before the test, 22.4 µL of 100 % TCA were added to each sample (supernatant of homogenized gills). After adding 100 % TCA, samples were vortexed for 1 minute and then centrifuged at 5000 rpm for 20 min. After centrifugation, 120 µL of supernatant was collected and put into a glass test tube; the remaining supernatant was put into a new 1.5 mL eppendorf tube. 120 µL of 0.1 M EDTA and 720 µL of the solution A were added into a glass tube with 120 µL of sample; the tubes were then covered with aluminium foil. Mixtures were boiled for 30 minutes and cooled at room temperature.

300 µL of each sample (in duplicates) and blank samples were transferred in the wells of 96 well microtrotiter plate and the absorbance was read at 532 nm. Blank was made of 120 µL of water, 120 µL of 0.1 M EDTA and 720 µL of the solution A.

3.6 Protein determination

Protein determination was carried out according to the Lowry method (Lowry, 1951). The necessary solutions for protein determination are listed in Table 3. I also prepared the necessary standard solutions with different BSA concentrations, which are listed in the Table 4.

Table 3: *Solutions for protein determination according to Lowry (*1951*) and the procedure for preparing them.*

Solution	Procedure
0.9 % NaCl Solution	Dilute 0.9 g of NaCl and 0.1 g of NaN_3 in 100 mL of dH_2O.
BSA Stock	Dilute 0.0625 g of BSA in 25 mL NaCl solution.
Solution A	Add 20 g Na_2CO_3 with 0.2 g of $KNaC_4H_4O_6 \cdot x\ 4H_2O$ to 1000 mL of 0.10 M NaOH.
Solution B	Add one drop of concentrate H_2SO_4 to 0.5 g CuSO4 to 100 mL of deionised water.
Solution A+B	Mix 0.1 mL of Solution B to 5 mL of Solution A.

Table 4: *BSA protein standard solutions.*

Standard	BSA concentration [µg/well]
Standard 1	6.25
Standard 2	12.50
Standard 3	25.00
Standard 4	50.00
Standard 5	100.00
Standard 6	150.00
Standard 7	200.00
Standard 8	250.00

285 µL of dH_2O and 15 µL of samples which were stored on ice were mixed in eppendorf tubes. Firstly, 100 µL dH_20 was added to the first column of microplate in duplicate (blank) followed by 100 µL of the protein standard solution BSA in duplicates. Then 100 µL of diluted samples (ratio 1:2) was added to wells in duplicates. 80 µL of Solution A+B was added into every well. The microplate was incubated for 10 minutes at room temperature and after incubation, I added Folin reagent (1:1). The next incubation lasted 30 minutes in the dark with agitation. When 30 minutes had passed the absorbance was read at 690 nm.

3.7 GST determination

GST activity was determined according to the method of Habig *et al*. (1974) adapted to the microplate following the procedure by Frasco and Guilhermino (2002). GST activity is calculated as the difference in absorbance between the control sample and the

mixture after the complete enzymatic conversion of substrate into the product. Firstly, I prepared the necessary solutions, which are listed in the Table 5.

Table 5: *Solutions for the GST assay and procedures for preparing them.*

Solution	Procedure
0.1 M Phosphate Buffer (GST Buffer)	Phosphate buffer was prepared with dilution of 3.4 g of KH_2PO_4 in 250 mL of dH_2O and 8.71 g of K_2HPO_4 in 500 mL of dH_2O. The solution of KH_2PO_4 was put into the solution of K_2HPO_4 until pH was 6.5.
10 mM GSH solution	Add 0.0154 g of GSH in 0.1 M phosphate buffer. The pH of the mixture was 6.5.
60 mM CDNB solution	Add 0.0121 g of CDNB in 1 mL of ethanol.
Reaction mixture	Reaction mixture was made of phosphate buffer, GSH solution and CDNB solution in a proportion of 4.95 (phosphate buffer): 0.9 (GSH solution): 0.15 CDNB solution.

In the microplate, I added 0.2 mL of the reaction mixture and 0.1 mL of the sample (supernatant from homogenized gills) and mixed it in the well. GST activity was measured immediately at the absorbance at 340 nm during 5 minutes in 20 second intervals. All reactions were performed in duplicates.

3.8 SOD determination

The SOD enzyme activity was determined according to McCord and Fridovich (1969) and adopted to the microplate assay.

Dilutions of sample (4 mg/mL) were made according to the results from protein determination in phosphate buffer. I added 450 µL of phosphate buffer to a stock solution of SOD (3000 U/mL). Necessary solutions for the determination of SOD activity are specified in Table 6 and SOD standard solutions in Table 7.

Table 6: *Solutions for the SOD assay and procedures for preparing them.*

Solution	Procedure
Solution of 50 mM phosphate buffer and 0.1 M Na_2EDTA	The solution was prepared in the following way: a) 8.95 g of Na_2HPO_4 + 9.305 g of Na_2EDTA was dissolved in 500 mL of dH_2O. b) 1.72 g of NaH_2PO_4 + 4.65 g of Na_2EDTA was dissolved in 250 mL of dH_2O. b) was added to a) until pH was 7.0.
0.1 mM Na_2EDTA	0.03722 g Na_2EDTA dissolved in 100 mL of H_2O.
2 mM KCN	130.24 mg of KCN dissolved in 1 L of dH_2O.
Solution A	55 mg of xantine in 5 mL of 0.001 M NaOH + 18 mg of cytochrome c in 50 mL of phosphate buffer.
Solution B	0.4 U/mL of xantine oxidase in 0.1 mM Na_2EDTA.

Table 7: *SOD standard solutions.*

Standard	SOD U/mL
Standard 6	40
Standard 5	30
Standard 4	20
Standard 3	10
Standard 2	5
Standard 1	2
Standard 0	1

I put 25 µL of sample (4 wells per sample) or standard in each well, and 25 µL of buffer and 50 µL of buffer for the blank sample. The determination of SOD was done in two wells with samples (duplicates) with 25 µL of 2 mM KCN. 200 µL of solution A was added in each well. In order to initiate a reaction, I added 50 µL of solution B in each well. The absorbance was read immediately with a spectrophotometer at 550 nm for 2 minutes in 20 second intervals.

3.9 Statistical analysis

Results were expressed as mean ± standard error of the mean. Statistical analysis was performed through analysis of the variance (one-way ANOVA) followed by Tukey's test. Statistical difference was designated as $p<0.05$.

4 RESULTS AND DISCUSSION

The results for LPO and GST activity for individuals *Pseudochondrostoma* sp. are listed in Appendix J. The results of measurements of LPO, GST and SOD activity for individuals *L. bocagei* are presented in Appendix K. Results are expressed as mean ± standard error of the mean.

4.1 Lipid peroxidation (LPO)

In the reaction, the amount of MDA was measured and expressed in nmol per miligram of protein. MDA is an end-product of LPO and can be used as an index of tissue oxidative stress.

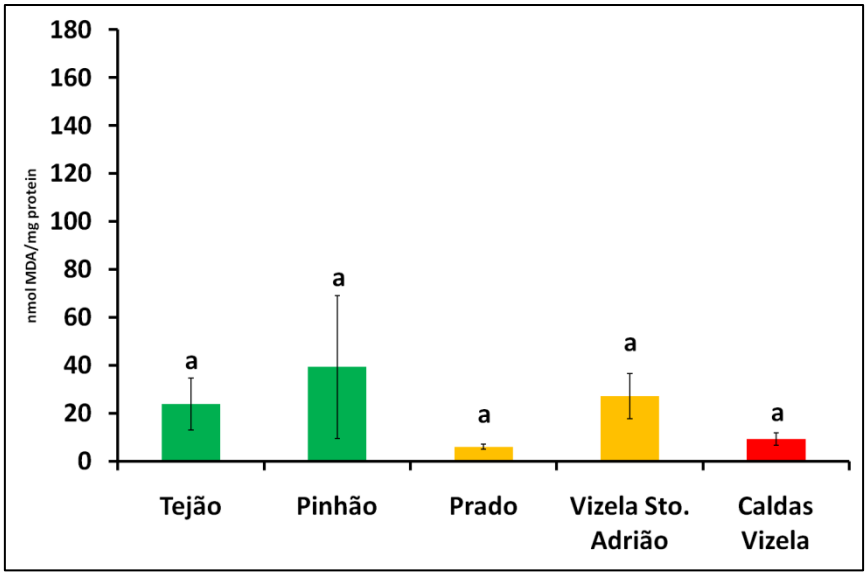

Figure 7: *Lipid peroxidation in the gill of* Pseudochondrostoma *sp. collected from five sampling sites with different levels of pollution (summer 2011).*

MDA levels were higher at reference sites Tejão (23.88 ± 10.86 nmol MDA/ mg protein) and Pinhão (39.36 ± 29.76 nmol MDA/ mg protein). MDA levels were lower at polluted sites Prado (5.99 ± 1.05 nmol MDA/ mg protein), Vizela Sto . Adrião (27.13 ± 9.49 nmol MDA/ mg protein) and Caldas Vizela (9.31 ± 2.62 nmol MDA/ mg protein), which is according to the WFD the most polluted site. It was expected that MDA levels would be higher at polluted sites and not at reference sites. Nevertheless, there was no observed statistical difference among the sites ($p>0.05$). When validating the results from site Pinhão, it is necessary to take into consideration that there were only two fish samples collected and that there was a big difference in the level of LPO among those two samples (high standard error of the means).

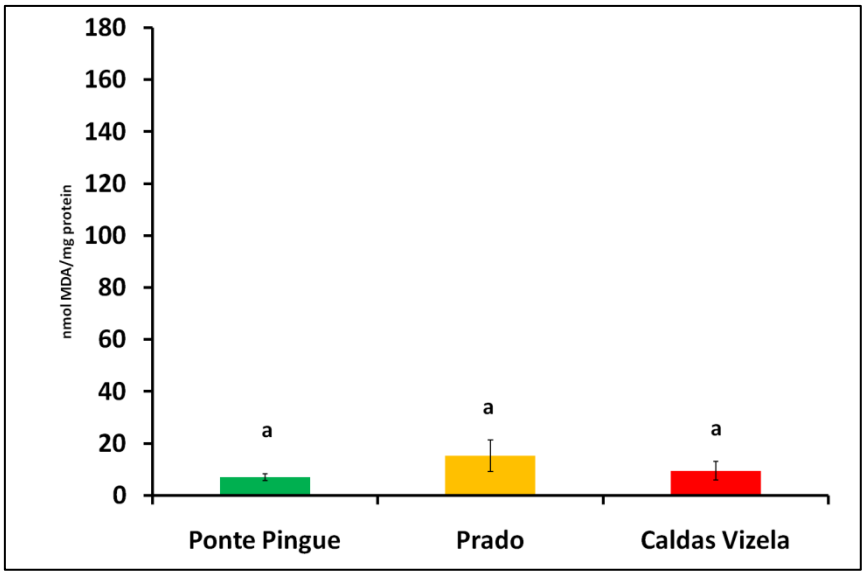

Figure 8: *Lipid peroxidation in the gill of* Pseudochondrostoma *sp. collected from three sampling sites with different levels of pollution (winter 2012).*

In winter 2012, MDA level contents were higher at Prado than at the reference site Ponte Pingue (7.02 ± 1.25 nmol MDA/ mg protein) and at the site Caldas Vizela (9.46 ± 3.64 nmol MDA/ mg protein). The lowest levels observed were at the site Ponte Pingue, which was expected as this site was a reference site. Among sites there was no observed statistical difference (p>0.05).

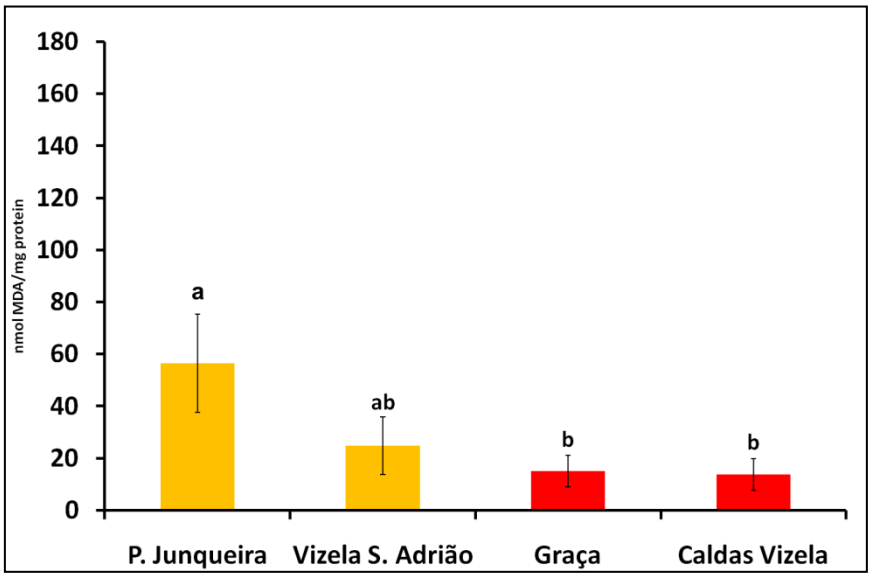

Figure 9: *Lipid peroxidation in the gill of* L. bocagei *collected from four sampling sites with different levels of pollution (summer 2011).*

MDA levels were the highest at the site Ponte Junqueira (56.48 ± 18.83 nmol MDA/ mg protein). LPO at other sites was significantly lower, the lowest being in the gills of *L. bocagei* collected from the site Caldas Vizela (13.70 ± 6.13 nmol MDA/ mg protein). There was an observed statistical difference among the site Ponte Junqueira and the sites Graça and Caldas Vizela. There was about 5-fold increase when comparing Ponte Junqueira to Graça and Caldas Vizela. There were no samples from reference

sites and there was no observed statistical difference (p>0.05) when comparing sites Ponte Junqueira and Vizela Sto. Adrião. There was also no observed statistical difference among sites Vizela Sto. Adrião, Graça and Caldas Vizela.

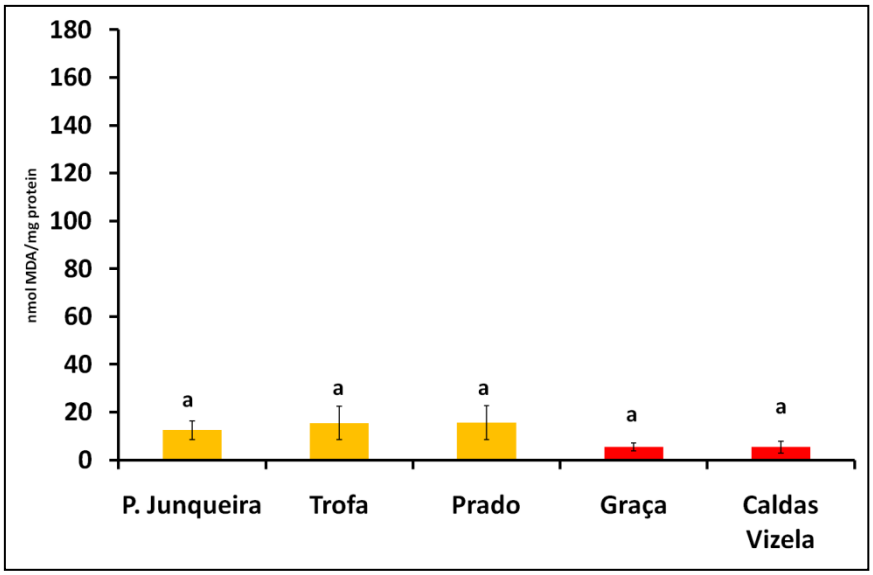

Figure 10: *Lipid peroxidation in the gill of* L. bocagei *collected from five sampling sites with different levels of pollution (winter 2012).*

LPO in gills of *L. bocagei* was the highest at sites Prado (15.66 ± 7.00 nmol MDA/ mg protein) and Trofa (15.52 ± 6.94 nmol MDA/ mg protein). At the site Ponte Junqueira MDA levels were lower (12.47 ± 3.94 nmol MDA/ mg protein) than at sites Prado and Trofa. The lowest observed values were at sites Graça (5.49 ± 1.74 nmol MDA/ mg protein) and Caldas Vizela (5.38 ± 2.41 nmol MDA/ mg protein). Even though there were differences in values among the sites, apparently there was no observed statistical difference among the sites (p>0.05).

The comparison of results between seasons revealed that LPO in the gills was significantly higher in summer 2011 compared to winter 2012 in both, *Pseudochondrostoma* sp. and *L. bocagei*. Several circumstances can promote antioxidant defence in fish. These factors can be age, phylogenetic position, feeding behaviour, and also environmental factors (daily or seasonal changes in temperature, dissolved oxygen, pollutants present in the water and the type of diet), which can either strengthen or weaken antioxidant defences (Martínez-Álvarez et al., 2005; Trenzado et al., 2006). In a study conducted by Ramsak and collaborators (2007), a seasonal variation in hepatic EROD activity in *Gobius niger* (Linnaeus, 1758*)* was observed. EROD activity was significantly higher in the non-spawning season in winter compared to the spawning period in spring. The difference was attributed to the reproduction cycle.

In the gills of *Pseudochondrostoma* sp. lower values of LPO were observed in winter, which can be observed from the site Caldas Vizela, as fish samples from this site were collected in both seasons. In the site Prado LPO was higher in the summer 2011. Abiotic factors could be the reason for higher levels of LPO in the summer, since the temperature is higher in the summer season.

L. bocagei was collected from three sites that were the same in summer 2011 and in winter 2012 and in the summer, values of LPO were higher at all sites. The biggest

difference was observed at the site Ponte Junqueira as LPO was almost six times higher in summer 2011 than in winter 2012. One of the reasons for the higher LPO values in summer could be related to the difference in temperature.

Amado et al. (2006) observed significant differences in LPO values between summer and winter in *Micropogonias furnieri* (Desmarest, 1823) in both contaminated and non-contaminated sites. Values were higher in the summer season. In the study conducted by Oliva et al. 2012, a significant difference was observed ($p<0.05$) in Senegalese sole (*Solea senegalensis,* Kaup, 1858) in the response of LPO between autumn and spring. The LPO values were higher in the autumn, which also showed a higher temperature than in spring.

Increases in environmental temperature result in the increase of oxygen consumption (Hochachka and Somero, 2002; Lushchak and Bognyukova, 2006). Temperature increase and enhanced oxygen consumption may promote ROS generation, oxidation of cellular components and a response of antioxidant and associated enzyme systems (Lushchak and Bognyukova, 2006). Furthermore, an increase in environmental temperature seriously reorganizes intermediary metabolism, changing chemicals composition, especially the relative levels of different kinds of lipids (Hochachka and Somero, 2002).

Pseudochondrostoma sp. and *L. bocagei* had similar LPO values in the gills at the site Caldas Vizela. The response in both species was higher in the summer when compared to winter.

The results showed that the values of LPO varied among sites. It is necessary to point out that there were only two samples obtained of *Pseudochondrostoma* sp*.* at the site Pinhão, and the value of LPO in those two samples varied extremely, meaning that the results from that site are not representative . Pinhão had the second highest value of LPO in the summer 2011 (39.36 ± 29.76 nmol MDA/ mg protein). Higher values were observed in the gills from the species *L. bocagei* at site Ponte Junqueira (56.48 ± 18.83 nmol MDA/ mg protein). Higher levels at the latter site were expected, since this site is considered to have a bad ecological status according to WFD. Surprisingly, sites with a bad ecological status, such as Caldas Vizela and Graça, had relatively low values of MDA levels. In a study with croakers (*Micropogonias furnieri*) (Amado et al., 2006), croakers from polluted sites had lower LPO values in the summer that the ones from non-polluted sites, which was also an unexpected result. According to researchers, a possible interpretation of the results is the inactivation of desaturases, enzymes that catalyze the addition of double bonds in a fatty acyl chain, playing a critical role in the biosynthesis of PUFAs (Pereira et al., 2003). Inactivation of desaturates could result in a reduction of LPO process in croakers from the polluted site due to a lower content of PUFAs in the cell membranes of these fish (Amado et al., 2006).

A possible explanation for the increase of LPO in *L. bocagei* and *Pseudochondrostoma* sp. in the summer was that the water temperature was responsible for the increased polyunsaturation of mitochondrial membranes in fish and raised rates of mitochondrial respiration, which would in turn enhance the formation of ROS, increase proton leak in favor of peroxidation of these membranes (Oliva et al., 2012). Furthermore, during the summer the water level is lower, which means that pollution is more concentrated, resulting thus in higher oxidative stress.

4.2 Glutathione-*S*-Tranferase (GST)

The change in the absorbance was recorded at 340 nm and the enzyme activity calculated as nanomoles of CDNB conjugate formed/min/mg protein using a molar extinction coefficient of $9.6x10^3$ $M^{-1}cm^{-1}$ of CDNB. As the height of the well is 0.9 cm, molar extinction coefficient of $8.64x10^3$ was used.

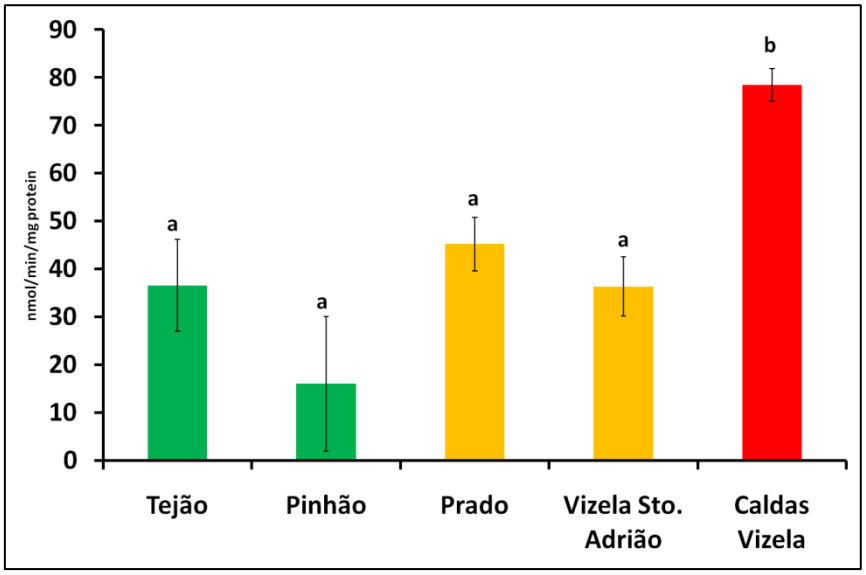

Figure 11: *Activity of GST in the gill of* Pseudochondrostoma *sp. collected from five sampling sites with different levels of pollution (summer 2011).*

The highest value of GST enzyme activity observed was at site Caldas Vizela (78.47 ± 3.44 nmol/min/mg protein). The lowest observed values were at reference sites Tejão (36.56 ± 9.61 nmol/min/mg protein) and Pinhão (16.02 ± 14.09 nmol/min/mg protein), and the values at sites with a poor ecological status , Prado and Vizela Sto . Adrião, were lower than at the site with a bad ecological status – Caldas Vizela – and higher than at reference sites. A statistical significance was observed between the site Caldas Vizela and the other locations. There was about 4.5-fold increase when comparing Caldas Vizela to Pinhão and about 2-fold increase compared to the sites Tejão and Vizela Sto. Adrião. The GST activity was 1.7 times lower in the site Prado in comparison to the GST activity in Caldas Vizela.

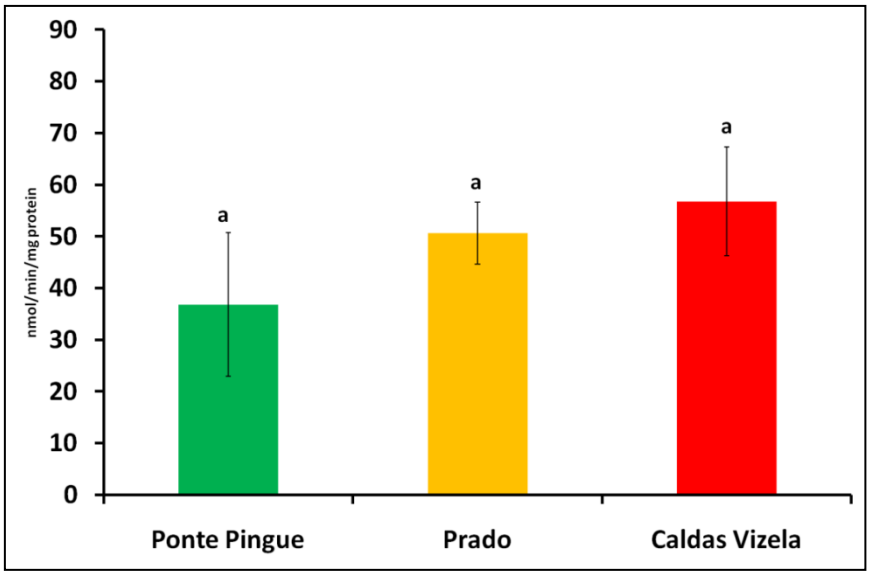

Figure 12: *Activity of GST in the gill of* Pseudochondrostoma *sp. collected from three sampling sites with different levels of pollution (winter 2012).*

The lowest observed value of GST enzyme activity was at the reference site Ponte Pingue (36.82 ± 13.90 nmol/min/mg protein), followed by site Prado (50.63 ± 6.02 nmol/min/mg protein). The highest observed value was at the site with a bad ecological status – Caldas Vizela (56.79 ± 10.57 nmol/min/mg protein). Among the sites there was no statistical difference in the value of GST activity ($p>0.05$).

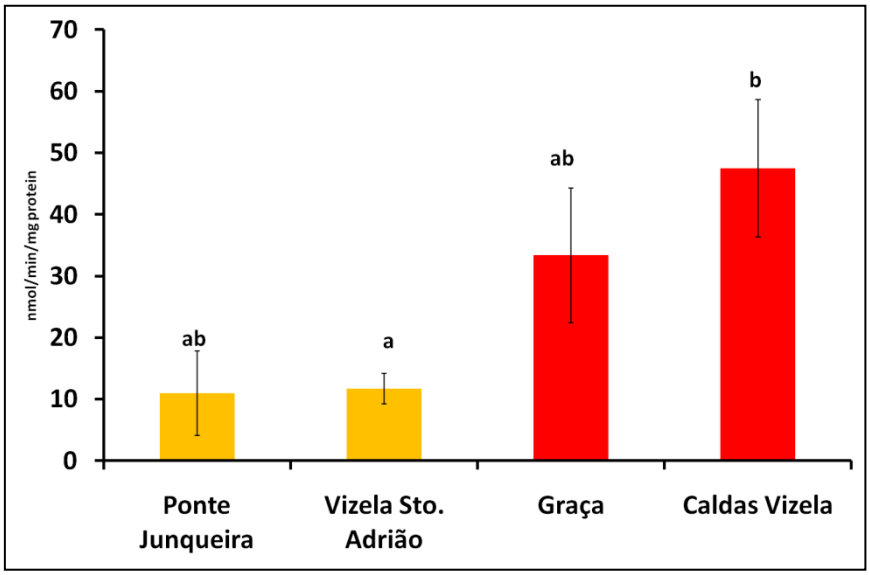

Figure 13: *Activity of GST in the gill of* L. bocagei *collected from four sampling sites with different levels of pollution (summer 2011).*

The highest observed values of GST activity were at sites with a bad ecological status, Graça (33.35 ± 10.94 nmol/min/mg protein) and Caldas Vizela (47.47 ± 11.16 nmol/min/mg protein). At the sites Ponte Junqueira and Vizela Sto. Adrião, values of enzyme activity were lower. The observed statistical difference ($p<0.05$) was between sites Caldas Vizela (47.47 ± 11.16 nmol/min/mg protein) and Vizela Sto . Adrião (11.68 ± 2.46 nmol/min/mg protein). The GST activity showed a 4-fold increase at the site Caldas Vizela when comparing it to the site Vizela Sto . Adrião. Even though there

was no observed statistical difference between sites Graça and Vizela Sto . Adrião, the GST activity was almost three times as high in Graça when comparing it to Vizela Sto . Adrião.

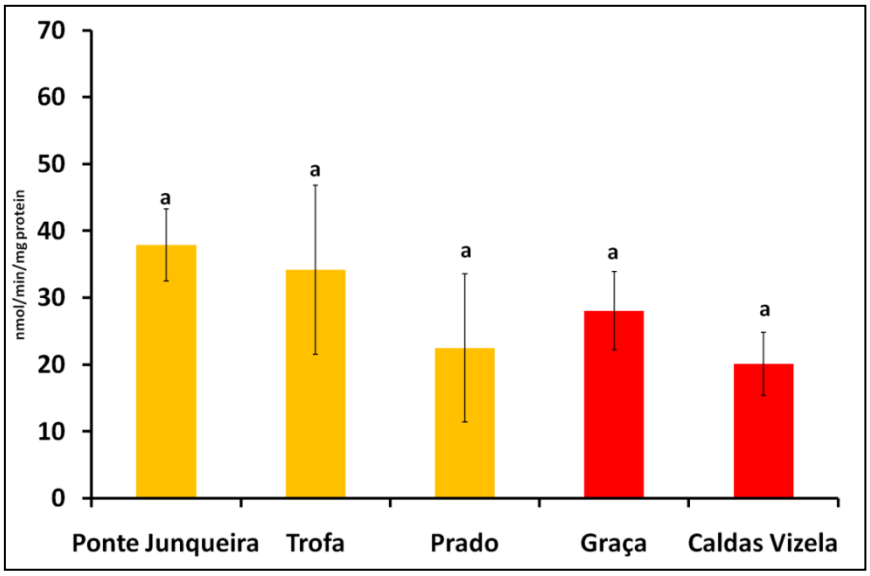

Figure 14: *Activity of GST in the gill of* L. bocagei *collected from five sampling sites with different levels of pollution (winter 2012).*

The GST activity was the highest at Ponte Junqueira (37.86 ± 5.40 nmol/min/mg protein), followed by Trofa, Graça and Prado. The lowest value was at the site with a bad ecological status, Caldas Vizela (20.12 ± 4.70 nmol/min/mg protein). There was no statistical difference among sites ($p>0.05$).

GST is a group of widely distributed enzymes that catalyzes the conjugation of reduced glutathione with compounds having reactive electrophilic groups, especially xenobiotics, such as metals and pesticides (Carvalho et al., 2012). GST levels in this study were in both species higher at polluted sites. Higher levels of GST at polluted sites were reported in a study with *Carassius gibelio (*Bloch, 1782), where GST levels differentiated the Dnieper River samples from Desna River and the control samples, since the values were higher in the Dnieper River samples (Tsangaris et al., 2011).

Comparing the results of GST activity among seasons, seasonal variation can be observed in *Pseudochondrostoma* sp., because the GST activity was higher in the summer. The seasonal variation in GST activity was also observed by Amado et al. (2006) in croakers, where the values were higher in warmer seasons. Seasonal adjustment in the antioxidant defence in thermo-conformers, like in most fish and invertebrates, suggest that this mechanism is a common adaptation in these species (Wilhelm Filho et al., 2001).

In *L. bocagei* the GST activity in the summer season was higher only at the polluted site Caldas Vizela (20.12 ± 4.70 nmol/min/mg protein). In a study with the fish *Geophagus brasiliensis* the GST activity at a non-polluted site was higher in the warmer period (Wilhelm Filho et al., 2001); meanwhile in this study the activity of GST in *L. bocagei* was higher at polluted sites (Graça and Caldas Vizela) in the summer period. What is problematic is the small number of samples obtained at the sites, since the number of fish samples is low and the standard error of the mean is high. The results in *L. bocagei* may not reflect the real picture of the sites, or the previous

ecological status had changed. Lower observed values may also be a result of tissue damage, since gills are directly in contact with environmental insults (Dautremepuits et al., 2009).

The comparison of the GST enzyme activity during the summer season in *Pseudochondrostoma* sp. and *L. bocagei*, revealed that the enzyme activities were higher in sites with a bad ecological status in both investigated species. The high levels of GST activity suggest the presence of organic contaminants (Ahmad et al., 2005). The presence of various xenobiotics, such as polycyclic aromatic hydrocarbons (Ahmad et al., 2005), mercury chloride (Monteiro et al., 2009) and pesticides is also known to induce GST activity.

4.3 Superoxide dismutase (SOD) activity

SOD activity was determined in the mitochondrial fraction as the degree of inhibition of cytochrome c reduction at 550 nm (McCord and Fridovich, 1969).

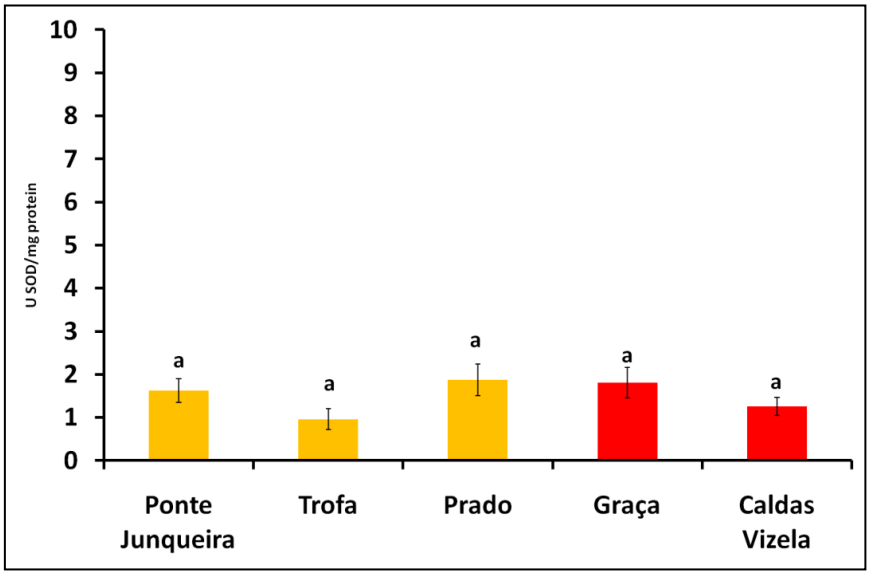

Figure 15: *Activity of SOD in the gill of* L. bocagei *collected from five sampling sites with different levels of pollution (winter 2012).*

Results of SOD activity were available only from winter 2012 for *L. bocagei.* There was no statistical difference among sites ($p>0.05$), and the highest value of SOD activity was observed at the sites Prado (1.87 ± 0.37 U SOD/mg protein) and Graça (1.81 ± 0.35 U SOD/mg protein), followed by Ponte Junqueira and Caldas Vizela. The lowest observed value was in the samples obtained from Trofa (0.96 ± 0.24 U SOD/mg protein).

SOD is one of the primary defences against oxygen toxicity caused by presence of metals. SOD is a metalloenzyme and plays a crucial role in the defence against ROS by transforming superoxide anions into hydrogen peroxide, which is detoxified by other enzymes. The SOD activity was small in the gills of *L. bocagei* and there were no significant differences among sites. The possible reason for the low enzyme activity could be the damage by oxidative modification (Carvalho et al., 2012). The low values of SOD activity may also be explained by the inhibition of SOD by hydrogen peroxide

(H_2O_2) or hydroxyl radicals ($OH^{-\bullet}$), since SOD catalyzes scavenging of superoxide anion radicals (Pandey et al., 2008).

Several field studies with different fish species have used the response of antioxidant and biotransformation as exposure biomarkers (van der Oost et al., 2003). Fish oxidative stress, as well as its antioxidant potential, differs in relation to species, habitat, and feeding behavior (Ahmad et al., 2004). In the literature, seasonal variations of oxidative stress biomarkers are influenced by abiotic factors, such as temperature and oxygen content (Sroda and Cossu-Leguille, 2011). Low oxygen concentration in water interferes with the fish population, causing death and inducing abnormalities in offspring. Temperature and pH affect the catalytic efficiency and binding capacity of the enzymes (Carvalho et al., 2012).

Both, *Pseudochondrostoma* sp. and *L. bocagei*, are benthic species and mainly bottom feeders (Kottelat and Freyhof, 2007), therefore higher levels oxidative stress enzymes activities may be due to the contaminants found in the river's sediments.

The comparison of antioxidant responses in *L. bocagei* and *Pseudochondrostoma* sp. can only be done for LPO and GST activity. At Site Caldas Vizela, specimens of *Pseudochondrostoma* sp. and *L. bocagei* were obtained both in the summer and in the winter season. *Pseudochondrostoma* sp. had similar values of LPO in both the summer and winter season (9.31 ± 2.62 nmol MDA/ mg protein in the summer and 9.49 ± 3.64 nmol MDA/ mg protein in the winter).

Higher LPO activities in the summer in *L. bocagei* could be a result of the spawning period, which for both species is in the spring season (April to June). In this study, LPO did not provide to be an adequate indicator of pollution. Findings were similar in the study by Pandey et al. (2003). The authors indicate that such findings could be a result of constant exposure of fish to pollutants, resulting in LPO reaching saturation point.

At site Caldas Vizela, GST activity was higher in *Pseudochondrostoma* sp. in both the summer and winter season when compared to GST activities in *L. bocagei*. At the same site, GST levels in both species were higher in the summer and lower in the winter season.

The results of GST activity in *Pseudochondrostoma* sp. can be compared to reference sites (Tejão, Pinhão and Ponte Pingue). In both, the summer and winter season; the lowest values of GST activities were at the reference sites and the highest values at the site with a bad ecological status (Caldas Vizela).

It was not possible to compare the activity of GST in *L. bocagei* to reference sites because these sites are located more upstream in the Ave and Douro basins. *L. bocagei* does not inhabit upper streams and is mainly found in lower and middle reaches of stream with a slow current. The most contaminated site according to the results is Caldas Vizela, which is consistent with the classification of its ecological status.

The main obstacle in validating the results was the small number of tested animals. In further biomonitoring, a higher number of animals must be tested along with physiological and chemical monitoring.

5 CONCLUSION

LPO activity was low in both *Pseudochondrostoma* sp. and *L. bocag*ei. Levels of LPO were higher in summer 2011 comparing to winter 2012. Several factors in the summer could be responsible for the higher level of LPO: higher temperatures, which cause increased rates of mitochondrial respiration and subsequently an enhanced formation of ROS resulting in the peroxidation of the membranes (Oliva et al., 2012). Also, higher temperatures means less dissolved oxygen and a lower oxygen concentration can be a reason for stress and can even cause death in fish populations (Carvalho et al., 2012).

The activity of the GST enzyme in *Pseudochondrostoma* sp. was higher in the summer. Lower activity of GST enzyme was recorded at sites with a good ecological status (Tejão and Pinhão) than with more polluted sites (with a bad ecological status). The opposite situation was observed in *L. bocagei* as GST enzyme activities were higher in winter 2012, except at the site with a bad ecological status – Caldas Vizela. Sampling site Caldas Vizela (bad ecological status) had the highest activity of GST enzymes in both species among all tested sampling sites in the summer. The high levels of GST activity suggest the presence of organic contaminants (Ahmad et al., 2005) probably due to agriculture and industrial discharges during the summer.

SOD activity was determined only in *L. bocagei* in winter 2012. The SOD activities values were low and we did not have samples from sites with a good ecological status and thus we were not able to make comparisons.

The hypothesis of the work was that the sites with a bad ecological status would have higher values of oxidative stress enzymes activities due to present pollutants. This was confirmed in *Pseudochondrostoma* sp. with the GST activity. The high levels of GST enzyme activity could indicate the presence of organic contaminants in the sites with a bad ecological status. For a more accurate interpretation of the results, the correlation analysis between water quality parameters and biological responses should be conducted. This is necessary as number of factors including habitat, nutrients, temperature, dissolved oxygen display influence on fish physiology (Pereira et al., 2013).

6 REFERENCES

- Ahmad I., Pacheco M., Santos M.A. 2004. Enzymatic and non-enzymatic antioxidants as an adaptation to phagocyte-induced damage in *Anguilla anguilla* L. following in situ harbor water exposure. *Ecotoxicology and Environmental Safety*, 57: 290-302

- Ahmad I., Oliveira M., Pacheco M., Santos M.A. 2005. *Anguilla anguilla* L. oxidative stress biomarkers responses to copper exposure with or without β-naphthoflavone pre-exposure. *Chemosphere,* 61: 267-275

- Ahmad I., Maria V.L., Oliveira M., Serafim A., Bebianno M.J., Pacheco M., Santos M.A. 2008. DNA damage and lipid peroxidation vs. protection responses in the gill of *Dicentrarchus labrax* L. from a contaminated coastal lagoon (Ria de Aveiro, Portugal). *Science of the Total Environment*, 406: 298-307

- Aksu Z. 2005. Application of biosorption for the removal of organic pollutants: a review. *Process Biochemistry*, 40: 997-1026

- Amado L .L., da Rosa C .E., Leite A .M., Moraes L ., Pires W .V., Leães Pinho G.L., Martins M.G.C., Robaldo R.B., Nery L.E.M., Monserrat J.M., Bianchini A., Martinez P.E., Geracitano L.A. 2006. Biomarkers in croakers *Micropogonias furnieri* (Teleostei: Sciaenidae) from polluted and non-polluted areas from the Patos Lagoon estuary (Southern Brazil): Evidence of genotoxic and immunological effects. *Marine Pollution Bulletin*, 52: 199-206

- Apel K., Hirt H. 2004. Reactive Oxygen Species: Metabolism, Oxidative Stress, and Signal Transduction. *Annual Review of Plant Biology,* 55: 373-399

- Cakir Y., Strauch S.M. 2005. Tricaine (MS-222) is a safe anaesthetic compound compared to benzocaine and pentobarbital to induce anaesthesia in leopard frogs (*Rana pipiens*). *Pharmacology Reports Online*, 57, 4: 467-474

- Cardoso da Silve M., Carmona Rodrigues A. 2004. Environmental indicators as tools for the management of estuaries-Methodology and case study of the Tejo estuary. *Journal of Coastal Conservation,* 10: 13-24

- Carvalho dos Santos C., Bernusso V.A., Selistre de Araújo H.S., Gaeta Espíndola E.L., Fernandes M.N. 2012. Biomarker responses as indication of contaminant effects in *Oreochromis niloticus*. *Chemosphere,* 89, 1: 60-69

- Cattrijsee A., Makwaia E.S., Dankwa, H.R., Hamerlynck O., Hemminga M.A. 1994. Nekton communities of an intertidal creek of a European estuarine brackfish marsh. *Marine Ecology-Progress Series*, 109: 195-208

- Cazenave J., Bacchetta C., Parma M.J., Scarabotti P.A., Wunderlin D.A. 2009. Multiple biomarkers in *Prochilodus lineatus* allowed assessing changes in the water quality of Salado River basin (Santa Fe, Argentina). *Environmental Pollution*, 157: 3025-3033

- Chubben N.H.P., Rietjens I.M.C.M., Wortelboer H., van Zanden J., van Bladeren P.J. 2001. The interplay of glutathione-related processes in antioxidant defense. *Environmental Toxicology and Pharmacology*, 10: 141-152

- Cortes R.M.V. 2009. Rio Douro: caracterizacão ecologica e valores ambientas. In: *as Aquas do Douro e Paiva: Edições Afrontamento*, pp. 201-214

- Da Rocha A .M., Salomão de Freitas D .P., Burns M., Vieira J.P., de la Torre F.R., Monserrat J.M. 2009. Seasonal and organ variation in antioxidant capacity, detoxifying competence and oxidative damage in freshwater and estuarine fishes from Southern Brazil. *Comparative Biochemistry and Physiology, Part C*, 150: 512-520

- Dautremepuits C., Marcogliese D.J., Gendron A.D., Fournier M. 2009. Gill and head kidney antioxidant processes and innate immune system responses of yellow perch (*Perca flavescens*) exposed to different contaminants in the St. Lawrence River, Canada. *Science of the Total Environment*, 407: 1055-1064

- Davies K.J.A. 1995. Oxidative stress: the paradox of aerobic life. *Biochemical Society Symposia*, Portland Press Limited, London, UK, 61: 1-31

- De Zwaart D. 1995. Monitoring water quality in the future, Volume 3: Biomonitoring. National Institute of Public Health and Environmental Protection (RIVM), Bilthoven, The Netherlands
  http://rivm.openrepository.com/rivm/bitstream/10029/10424/1/719102049.pdf
  19.7.2013

- De Zwaart L.L., Meerman J.H.N., Commandeur J.N.M., Vermeulen N.P.E. 1999. Biomarkers of free radical damage: Applications in experimental animals and in humans. *Free Radical Biology & Medicine*, 26: 202-226

- Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water polici (Water Framework Directive (WFD)). *Official Journal of the European Union (L series)*, 327: 1-73

- Dröge W. 2002. Free radicals in the physiological control of cell function. *Physiological Reviews*, 82: 47-95

- Evans D.H., Piermarini P.M., Choe K.P. 2005. The Multifunctional Fish Gill: Dominant Site of Gas Exchange, Osmoregulation, Acid-Base Regulation, and Excretion of Nitrogeneous Waste. *Physiological Reviews*, 85: 97-177

- Fenoglio C., Boncompagni E., Fasola M., Gandini C., Comizzoli S., Milanesi G., Barni S. 2005. Effects of environmental pollution on the liver parenchymal cells and Kupffer-melanomacrophagic cells of the frog *Rana esculenta*. *Ecotoxicology and Environmental Safety*, 60: 259-268

- Frasco M.F., Guilhermino L. 2002. Effects of dimethoate and beta-naphtoflavone on selected biomarkers of *Poecilia reticulata*. *Fish Physiology and Biochemistry,* 26: 149-156

- Fernandes C., Fontaínhas-Fernandes A., Ferreira M., Salgado M.A. 2008. Oxidative Stress Response in Gill and Liver of *Liza Saliens*, from the Esmoriz-Paramos Coastal Lagoon, Portugal. *Archives of Environmental Contamination and Toxicology*, 55: 262-269

- Gomez M.J., Herrerra S., Solé D., García-Calvo E., Fernández-Alba A.R. 2012. Spatio-temporal evaluation of organic contaminants and their transformation products along a river basin affected by urban, agricultural and industrial pollution. *Science of the Total Environment,* 420: 134-145

- Gonçalves E.P.E., Boaventura R. 1991. Sediments as indicators of the river Ave contamination by heavy metals. In: *Integrated Approaches to Water Pollution Problems.* Bau J., Ferreira J.P.L., Henriques J.D., Raposo J.O. (Eds), Elsevier, London, pp. 209-218

- Gupta V.K., Sharma S.K. 2006. Plants as natural antioxidants. *Natural Product Radiance*, 5, 4: 326-334

- Habig W.H., Pabst M.J., Jakoby W.B. 1974. Glutathione-*S*-Transferases: The first enzymatic step in mercapturic acid formation*. The Journal of Biological Chemistry*, 249, 22: 7130-7139

- Hao L., Chen L. 2012. Oxidative stress responses in different organs of carp (*Cyprinus carpio*) with exposure to ZnO nanoparticles. *Ecotoxicological and Environmental Safety,* 80: 103-110

- Harman D. 1956. Aging: a theory based on free radical and radiation chemistry. *Journal of Gerontology*, 11, 3: 298-300

- Henzová M., Kiss Deér A., Filla A., Komlósi V., Mink J. 2008. Effects of Cu^{2+} and Pb^{2+} on different fish species: Liver cytochrome P450-dependent monooxygenase activities and FTIR spectra*. Comparative Biochemistry and Physiology, Part C*, 148: 53-60

- Hochachka P.W., Somero G.N. 2002. Biochemical Adaptation: Mechanism and process in Physiologial Evolution. Oxford University Press, Oxford, UK, pp. 409-435.

- Holt E.A., Miller S.W. 2011. Bioindicators: Using Organisms to Measure Environmental Impacts. *Nature Education Knowledge*, 2, 2: 8

- Islam S., Tanaka M. 2004. Impacts of pollution on coastal and marine ecosystems including coastal and marine fisheries and approach for management: a review and synthesis. *Marine Pollution Bulletin*, 48: 624-649

- Kappus H., Sies H. 1981. Toxic drug effects associated with oxygen metabolism: Redox cycling and lipid peroxidation. *Experientia*, 37, 12: 1233-1358

- Kime D. E. 1995. The effects of pollution on reproduction in fish. *Reviews in Fish Biology and Fisheries*, 5: 52-96

- Kohen R., Nyska A. 2002. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicologic Pathology*, 30, 6: 620-650

- Kottelat M., Freyhof J. 2007. Handbook of European Freshwater Fishes. Kottelat, Cornol, Switzerland and Freyhof, Berlin, Germany, pp. 135-136; 232-233

- Lackner R. 1998. Oxidative Stress. In: *Fish by Environmental Pollutants*. Braunbeck T., Hinton D.E., Streit B. (Eds). Fish Ecotoxicology, Berlin, Germany, pp: 203-224

- Lemaire P., Viarengo A., Canesi L., Livingstone D.R. 1993. Pro-oxidant and oxidant processes in gas gland and other tissues of cod (*Gadus morhua*). *Journal of Comparative Physiology, Part B*, 163: 477-486

- Levine R.L., Wehr N., Williams J.A., Stadtman E.R., Shacter E. 2000. Determination of Carbonyl Groups in Oxidized Proteins. *Methods in Molecular Biology*, 99, I: 15-24

- Li L., Zheng B., Liu L. 2010. Biomonitoring and Bioindicators Used for River Ecosystems: Definitions, Approaches and Trends. *Procedia Environmental Sciences*, 2: 1510-1524

- Linde-Arias A.R., Inácio A.F., Novo L.O., De Albuerquerque C., Moreira J.C. 2008. Multibiomarker approach in fish to assess the impact of pollution in a large Brazilian river, Paraiba do Sul. *Environmental Pollution*, 156: 974-979

- Livingstone D.R., Forlin L.,George S. 1994. Molecular biomarkers and toxic consequences of impact by organic pollution in aquatic organisms. In*: Water Quality and Stress Indicators in Marine and Freshwater Systems: Linking Levels of Organization.* Sutcliffe D.W. (Ed.), Freshwater Biological Association, Ambleside, UK, pp. 1171-1254

- Lowry O.H., Rosebrough N.J., Farr L.A., Randall J.R. 1951. Protein measurement with Folin phenol reagent. *The Journal of Biological Chemistry*, 193(1): 265-275

- Lushchak V.I., Bagnyukova T.V. 2006. Temperature increase results in oxidative stress in goldfish tissues. 1. Indices of oxidative stress. *Comparative Biochemistry and Physiology, Part C*, 143: 30-35

- Lushchak V.I. 2011. Environmentally induced oxidative stress in aquatic animals. *Aquatic Toxicology*, 101: 279-294

- Madureira T.A., Barreiro J.C., Rocha M.J., Rocha E., Cass Q.B., Tiritan M.E. 2010. Spatiotemporal distribution of pharmaceuticals in the Douro River estuary (Portugal). *Science of the Total Environment*, 408: 5513-5520

- Madureira T.V., Rocha M.J., Cruzeiro C., Rodrigues I., Monteiro R.A.F., Rocha E. 2012. The toxicity potential of pharmaceuticals found in the Douro River estuary (Portugal): Evaluation of impacts on fish liver, by histopathology,

stereology, vitellogenin and CYP1A immunohistochemistry, after sub-acute exposures of the zebrafish model. *Environmental Toxicology and Pharmacology*, 34: 34-45

- Marques dos Santos D.C., Pinto da Matta S.L., Alves de Oliveira J., Abdala Dergam dos Santos J. 2011. Histological alterations in gills of *Astyanax* aff, *bimaculatus* caused by acute exposition to zinc. *Experimental and Toxicologic Pathology,* 64, 7-8: 861-866

- Martínez-Álvarez R.M., Morales A.E., Sanz A. 2005. Antioxidant defences: Biotic and abiotic factors. *Reviews in Fish Biology and Fisheries*, 15: 75-88

- McCord J.M., Fridovich I. 1969. Superoxide dismutase: An enzymatic Function for Erythrocuprein (Hemocuprein). *The Journal of Biological Chemistry,* 244, 22: 6049-6055

- Metcalfe J.L. 1989. Biological water quality assessment of running waters based on macroinvertebrate communities: History and present status in Europe. *Environmental Pollution*, 60, 1-2: 101-139

- Monteiro M., Quintaneiro C., Nogueira A.J.A., Morgado F., Soares A.M.V.M., Guilhermino L. 2007. Impact of chemical exposure on the fish *Pomatoschistus microps* (Krøyer, 1838) in estuaries of the Portuguese Northwest coast. *Chemosphere*, 66: 514-522

- Monteiro S.M., Rocha E., Mancera J.M., Fontaínhas-Fernandes A., Sousa M. 2009. A stereological study of copper toxicity in gills of *Oreochromis niloticus. Ecotoxicology and Environmental Safety*, 72: 213-223

- Naigaga I., Kaiser H., Muller W.J., Ojok L., Mbabazi D., Magezi G., Muhumuza E. 2011. Fish as bioindicators in aquatic environmental pollution assessment: A case study in Lake Victoria wetlands, Uganda. *Physics and Chemistry of the Earth*, 36: 918-928

- National Research Council (NRC): Biological Committee on Biological Markers. 1987. Biological Markers in Environmental Health Research. *Environmental Health Perspectives*, 74: 3-9

- Niki E., 2000. Lipid peroxides. In: *Experimental protocols for reactive oxygen and nitrogen species*. Tamiguchi J., Gutteridge J.M.C (Eds), Oxford University Press, Oxford, UK, pp. 156-160

- Nordberg J., Arnér E.S.J. 2001. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radical Biology & Medicine*, 31, 11: 1287-1312

- Ohe T., Watanabe T., Wakabayashi K. 2004. Mutagens in surface waters: A review. *Mutation Research*, 567: 109-149

- Oliva M., Vicente J.J., Gravato C., Guilhermino L. 2012. Oxidative stress biomarkers in Senegal sole, *Solea senegalensis*, to assess the impact of heavy

metal pollution in a Huelva estuary (SW Spain): Seasonal and spatial variation. *Ecotoxicology and Environmental Safety,* 75: 151-162

- Oliveira R.E.S., Lima M.M.C.L., Vieira J.M.P. 2005. An indicator system for water quality in river basins. *The Fourth Inter-Celtic Colloquium on Hydrology and Management of Water Resources*, Guimarães, Portugal (July 11-14)

- Oliveira M., Ahmad I., Maria V.L., Pacheco M., Santos M.A. 2010. Monitoring pollution of coastal lagoon using *Liza aurata* kidney oxidative stress and genetic endpoints: an integrated biomarker approach. *Ecotoxicology*, 19: 643-653

- Oruc E.O., Sevgiler Y., Uner N. 2004. Tissue-specific oxidative responses in fish exposed to 2,4-D and azinphosmethyl. *Comparative Biochemistry and Physiology, Part C*, 137: 43-51

- Ozmen M., Ayas Z., Güngördü A., Ekmekci G.F., Yerli S. 2008. Ecotoxicological assessment of water pollution in Sariyar Dam Lake, Turkey. *Ecotoxicology and Environmental Safety*, 70: 163-173

- Pandey S., Parvez S., Sayeed I., Haque R., Bin-Hafeez B., Raisuddin S. 2003. Biomarkers of oxidative stress: a comparative study of river Yamuna fish *Wallago attu* (Bl. & Schn.). *Science of the Total Environment*, 309: 105-115

- Pandey S., Parvez S., Ahamd Ansari R., Ali M., Kaur M., Hayat F., Ahmad F., Raisuddin S. 2008. Effects of exposure to multiple trace metals on biochemical, histological and ultrastructural features of gills of a freshwater fish, *Channa punctata* (Bloch). *Chemico-Biological Interactions*, 174: 183-192

- Pappa S., Skulachev V.P. 1997. Reactive oxygen species, mitochondria, apoptosis and aging. *Molecular and Cellular Biochemistry*, 174: 305-319

- Parvez S., Raisuddin S. 2005. Protein carbonyls: novel biomarkers of exposure to oxidative stress-inducing pesticides in freshwater fish Channa punctata (Bloch). *Environmental Toxicology and Pharmacology*, 20: 112-117

- Parvez S., Pandey S., Ali M., Raisuddin S. 2006. Biomarkers of oxidative stress in Wallago attu (Bl. & Sch.) during and after a fish-kill episode at Panipat, India. *Science of the Total Environment*, 368: 627-636

- Peng S., Chen L., Qin J.G., Hou J., Yu N., Long Z., Ye J., Sun X. 2008. Effects of replacement of dietary fish oil by soybean oil on growth performance and liver biochemical composition in juvenile black seabream, *Acanthopagrus schlegeli. Aquaculture*, 276: 154-161

- Pereira S., Pinto A.L., Cortes A., Fontáinhas-Fernandes A., Coimbra A.M, Monteiro S.M. 2013. Gill histopathological and oxidative stress evaluation in native fish captured in Portuguese northwestern rivers. *Ecotoxicology and Environmental Safety*, 90: 157-166

- Pereira S.L., Leoonard A.E., Mukerji P. 2003. Recent advances in the study of fatty acid desaturases from animals and lower eukaryotes. *Prostaglandines, Leukotrienses and Essential Fatty Acids,* 68: 97-106

- Playle R.C. 1998. Modelling metal interactions at fish gills. *The Science of the Total Environment*, 219: 147-163

- Powell M.D., Speare D.J., Burka J.F. 1992. Fixation of mucus on rainbow trout (*Oncorhynchus mykiss* Walbaum) gills for light and electron microscopy. *Journal of Fish Biology*, 41, 5: 813-824

- Ramšak A., Stopar K., Sepčić K., Berden-Zimec M., Bajt O., Malej A. 2007. Reflection of hydrocarbon pollution on hepatic EROD activity in the black goby (*Gobius niger*). *Environmental Toxicology and Pharmacology*, 24: 304-310

- Rice J. 2003. Environmental health indicators. *Ocean & Coastal Management*, 46: 235-259

- Robalo J.I., Almada V.C., Levy A., Doadrio I. 2007. Re-examination and phylogeny of the genus *Chondrostoma* based on mitochondrial and nuclear data and the definition of 5 new genera. *Molecular Phylogenetics and Evolution*, 42: 362-372

- Sazuka Y., Tanizawa H., Takino Y. 1989. Effect of Adriamycin on the Activities of Superoxide Dismutase, Glutathione peroxidase and Catalase in Tissues of Mice. *Japanese Journal of Cancer Research*, 80: 89-94

- Singh N.P., Ogburn C.E., Wolf N.S., van Belle G., Martin G.M. 2001. DNA double-strand breaks in mouse kidney cells with age. *Biogerontology,* 2: 261-270

- Soares H.M.V.M., Boaventura R.A.R., Machado A.A.S.C., Esteves da Silva J.C.G. 1999. Sediments as monitors of heavy metal contamination in the Ave river basin (Portugal): multivariate analysis of data. *Environmental Pollution*, 105: 311-323

- Soengas J.L., Aldegunde M. 2002. Energy metabolism of fish brain. *Comparative Biochemistry and Physiology, Part B*, 131: 271-296

- Soininen J., Könönen K. 2004. Comparative study for monitoring South-Finnish rivers and streams using macrovertebrate and benthic diatom community structure. *Aquatic Ecology*, 38: 63-75

- Solé M., Lobera H., Aljinovic B., Ríos J., García de la Parra L.M., Maynou F., Cartes J.E. 2008. Cholinesterase activities and lipid peroxidation levels in muscle from shelf and slope dwelling fish from the NW Mediterranean: Its potential use in pollution monitoring. *Science of the Total Environment*, 402: 306-317

- Soldán P. 2003. Toxic risk of surface water pollution – six years of experience. *Environmental International*, 28: 677-682

- Sroda S., Cossu-Leguille C. 2011. Seasonal variability of antioxidant biomarkers and energy reserves in the freshwater gammarid (*Gammarus roeseli). Chemosphere*, 83: 538-544

- Stegeman J.J., Hahn, M.E. 1994. Biochemistry and molecular biology of monooxygenase: current directions on forms, functions, and regulation of cytochrome P450 in aquatic species. In: *Aquatic* toxicology: Molecular, Biochemical and Cellular perspectives. Malins, D.C., Ostrander, G.K. (Eds*),* Lewis Publishers, CRC press, Boca Raton, pp. 87-206

- Suter G.W. 1990. Use of biomarker in ecological risk assessment. In: *Biomarkers of Environmental Contamination*. McCharthy J.F., Shugart L.R. (Eds), Lewis Publisher, Boca Raton, Florida, USA, pp. 419-428

- Tew K.D. 1994. Glutathione-associated Enzymes in Anticancer Drug Resistance. *Cancer Research*, 54: 4313-4320

- Torres M.A., Barros M.P., Campos S. C. G., Pinto E., Rajamani S., Sayre R.T., Colepicolo P. 2008. Biochemical biomarkers in algae and marine pollution: A review. *Ecotoxicology and Environmental Safety,* 71: 1-15

- Trenzado C., Hidalgo C.M., García-Gallego M., Morales A.E., Furné M., Domezain A., Domezain J., Sanz A. 2006. Antioxidant enzymes and lipid peroxidation in sturgeon *Acipenser naccarii* and trout *Oncorhynchus mykiss*. A comparative study. *Aquaculture*, 254: 758-767

- Tsangaris C., Vergolyas M., Fountolaki E., Goncharuk V.V. 2011. Genotoxicity and oxidative stress biomarkers in Carassius gibelio as endpoints for toxicity testing of Ukrainian polluted river waters. *Ecotoxicology and Environmental Safety*, 74: 2240-2244

- United Nations 1982. United Nations Convention on the Law of Sea http://www.un.org/Depts/los/convention_agreements/texts/unclos/unclos_e.pdf 9.6.2012

- USA Senate 2011. Federal Water Pollution Control Act (Clean Water Act). 33 U.S:C § 1251 et seq., Section 502(14), pp. 117 (as in effect January 4, 2011).

- U.S. Environmental Protection Agency (EPA) 2005. Protecting water quality from Agricultural runoff (Fact sheet). http://www.epa.gov/owow/NPS/Ag_Runoff_Fact_Sheet.pdf 10.6.2012

- U.S. Environmental Protection Agency (EPA) 2012. What is nonpoint source pollution? http://water.epa.gov/polwaste/nps/whatis.cfm 10.6.2012

- Valavanidis A., Vlahogianni T., Dassenakis M., Scoullos M. 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicology and Environmental Safety*, 64: 178-189

- Van der Oost R., Beyer J., Vermeulen, N.P.E. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology*, 13: 57-149

- Van Ginneken V., Nieween M., Van Eersel R., Van den Thilht G., Addink A. 1996. Neurotransmitter levels and energy status in brain of fish species with and without the survival strategy of metabolic depression. *Comparative Biochemistry and Physiology, Part A,* 114, 2: 189-196

- Viarengo A., Canesi L., Pertica M., Poli G., Moore M.N., Orunesu M. 1990. Heavy metal effects on lipid peroxidation in the tissues of *Mytilus galloprovincialis*. *Comparative Biochemistry and Physiology, Part C: Comparative Pharmacology*, 97: 37-42

- Viarengo A., Canesi L. 1991. Mussesls as biological indicators of pollution. *Aquaculture*, 94: 225-243

- Vicentini C.A., Franceschini-Vicentini I.B., Bombonato M.T.S., Bertolucci B., Lima S.G., Santos A.S. 2005. Morphological Study of the Liver in the Teleost *Oreochromis niloticus*. *International Journal of Morphology*, 23, 3: 211-216

- Wilhelm Filho D., Torres M.A., Tribess T.B., Pedrosa R.C., Soares C.H.L. 2001. Influence of season and pollution on the antioxidant defenses of the cichlid fish acará (*Geophagus brasiliensis*). *Brazilian Journal of Medical and Biological Research*, 34: 719-726

- Williams C. 1996. Combating marine pollution from land-based activities: Australian initiatives. *Ocean & Coastal Management*, 33: 87-112

- Winterbourn C.C., Hampton M.B. 2008. Thiol chemistry and specifity in redox signaling. *Free Radical Biology & Medicine*, 45: 549-561

- Zhou Q., Zhang J., Fu J., Shi J., Jiang G. 2008. Biomonitoring: An appealing tool for assessment of metal pollution in the aquatic ecosystem. *Analytica Chimica Acta*, 606: 135-150

APPENDIXES

APPENDIX A

Chemicals used for protein determination

Chemical	Manufacturer
KH_2PO_4	Riedel-de Haën, Seelze, Germany
K_2HPO_4	Sigma-Aldrich, St. Luis, USA
Na_2EDTA	Pharmacia Biotech, Uppsala, Sweden

APPENDIX B

Chemicals used for TBARS assay

Chemical	Manufacturer
TCA (Trichloro acetic acid)	Sigma-Aldrich, St. Luis, USA
Na_2EDTA	Pharmacia Biotech, Uppsala, Sweden
2-TBA	Sigma-Aldrich, St. Luis, USA
BHT	Sigma-Aldrich, St. Luis, USA
NaOH	Pronalab, Lisboa, Portugal

APPENDIX C

Chemicals used for GST determination

Chemical	Manufacturer
NaCl	Absolute, Rochester Hills, USA
Sodium azide (NaN_3)	Sigma-Aldrich, St. Luis, USA
Bovine Serum Albumine	Sigma-Aldrich, St. Luis, USA
Na_2CO_3	Riedel-de Haën, Seelze, Germany
Sodium-Potassium Tartate	Sigma-Aldrich, St. Luis, USA
H_2SO_4	Sigma-Aldrich, St. Luis, USA
$CuSO_4$	Sigma-Aldrich, St. Luis, USA

Folin reagent	Merck, Darmstadt Germany
CDNB	Sigma-Aldrich, St. Luis, USA
KH_2PO_4 (Potassium phosphate monobasic)	Riedel-de Haën, Seelze, Germany
K_2HPO_4 (Potassium phosphate dibasic)	Sigma-Aldrich, St. Luis, USA
Ethanol	Merck, Darmstadt, Germany

APPENDIX D

Chemicals used for SOD determination

Chemical	Manufacturer
SOD (from bovine liver)	Sigma-Aldrich, St. Luis, USA
KCN	Sigma-Aldrich, Steinheim, Germany
Na_2EDTA	Pharmacia Biotech, Uppsala, Sweden
Xantine	Sigma-Aldrich, St. Luis, USA
Xantine oxidase	Sigma-Aldrich, St. Luis, USA
Cytochrome c	Sigma-Aldrich, St. Luis, USA
NaH_2PO_4	Sigma-Aldrich, St. Luis, USA

APPENDIX E

Geographical coordinates of the sampling locations

Sampling site	Geographical coordinates
Ponte de Pingue	41°27'2.33"N; 8°9'26.13"W
Tejão	41°21'52.58"N; 7°55'41.08"W
Pinhão	41°20'38.26"N; 7°35'25.67"W
Ponte Junqueira	41°23'18.01"N; 8°41'24.66"W
Prado	41°36'2.93"N; 8°28'15.99"W

Trofa	41°20'44.30"N; 8°33'50.13"W
Vizela Sto. Adrião	41°22'14.65"N; 8°16'29.75"W
Graça	41°21'59.57"N; 8°41'49,83"W
Caldas de Vizela	41°22'24.62"N; 8°18'38.19"W

APPENDIX F

Basic information for *Pseudochondrostoma* sp. from summer fishing 2011

Site	**Length**		**Weight**		
	Number of fish	**Total length (cm)**	**Body length (cm)**	**Body mass(g)**	**Gill mass (g)**
Ponte Pingue	**11**	11.0	9.4	12	0.001
	12	11.0	9.5	15	0.013
	13	12.0	9.3	12	No sample
Prado	**16**	22.7	19.4	113	0.074
	17	19.0	16.0	72	0.168
	18	23.4	20.0	123	0.232
	19	18.2	15.4	47	0.113
	20	18.5	16.0	60	0.066
	21	18.1	15.6	61	0.190
	22	19.5	16.5	68	0.062
	23	18.7	16.0	55	0.112
	24	20.0	17.0	85	No sample
	25	20.0	16.8	78	0.115
Vizela Sto. Adrião	**51**	11.4	9.7	15	0.095
	52	20.0	17.0	94	0.099
	53	18.8	16.3	81	0.024
	54	18.0	15.3	64	0.085
	55	16.0	14.3	53	0.118
Tejão	**61**	16.1	13.3	40	0.144
	62	15.1	12.5	33	0.081
	63	13.7	11.5	29	0.094
	64	14.7	12.4	27	0.023
	65	14.4	12.2	27	0.110
Pinhão	**68**	13.9	11.7	29	0.025
	69	12.0	10.5	15	0.028
Caldas Vizela	**84**	19.0	16.7	86	0.358
	85	20.3	17.5	87	0.283
	86	20.5	17.3	87	0.400

	90	23.0	20.1	148	0.484
	91	20.7	17.8	97	0.445

APPENDIX G

Basic information for *Pseudochondrostoma* sp. from winter fishing 2012

Site	**Length**			**Weight**	
	Number of fish	**Total length (cm)**	**Body Length (cm)**	**Body mass (g)**	**Gill mass (g)**
Caldas Vizela	123	24.3	22.4	149	No sample
	127	14.6	13.6	34	0.138
	128	13.2	12.0	24	0.082
	129	13.9	13.0	31	0.317
	130	14.0	13.0	33	0.051
Prado	147	19.5	18.3	74	0.199
	148	18.4	17.0	58	0.170
	149	17.5	16.5	55	0.011
	150	17.5	16.5	49	0.188
	151	16.4	15.4	42	0.087
Ponte Pingue	159	11.5	11.0	17	0.064
	160	12.6	12.3	28	0.090
	161	11.8	11.5	19	0.064

APPENDIX H

Basic information for *L. bocagei* from summer fishing 2011

Site	**Length**			**Weight**	
	Number of fish	**Total Length (cm)**	**Body Length (cm)**	**Total mass (g)**	**Gill mass (g)**
Ponte Junqueira	**26**	20.0	16.5	54	0.100
	27	12.7	11.0	19	0.040
	28	12.7	9.5	20	0.018
	29	12.5	10.8	18	0.022
	30	14.0	12.0	27	0.046
	31	12.3	10.4	17	0.050
	32	12.0	10.0	11	0.021
	33	12.5	10.5	15	No sample
	34	11.0	9.0	12	0.013
	35	11.2	9.5	14	0.019
Vizela Sto. Adrião	**46**	25.5	21.5	166	0.135

	47	28.0	23.3	193	0.301
	48	21.1	19.0	83	0.079
	49	27.3	25.0	200	0.369
	50	23.3	19.6	130	0.090
Graça	**75**	29.5	27.0	282	0.437
	76	26.5	22.0	164	0.156
	77	26.0	21.7	171	0.441
	78	33.2	27.6	330	0.415
	80	23.5	19.5	90	0.100
	82	29.5	24.0	235	0.590
Caldas Vizela	**87**	15.7	13.7	48	0.306
	88	22.0	19.2	106	0.316
	89	18.0	15.7	63	0.268
	92	20.7	18.0	94	0.637
	93	20.2	17.7	91	0.519

APPENDIX I

Basic information for *L. bocagei* from winter fishing 2012

Site	**Length**		**Weight**		
	Number of fish	**Total length (cm)**	**Body length (cm)**	**Total mass (g)**	**Gill mass (g)**
Ponte Junqueira	101	17.4	15.7	44	0.172
	102	15.3	13.9	35	0.218
	103	12.8	11.7	16	0.066
	104	19.3	17.5	73	0.206
	105	30.4	28.0	249	0.205
	106	23.5	21.5	120	0.209
	107	21.0	19.0	91	0.352
	108	18.0	16.9	61	0.210
	109	22.6	20.6	111	0.247
	110	20.0	18.0	76	0.223
Graça	111	30.0	27.0	245	0.355
	112	21.8	19.8	95	0.435
	113	23.2	21.2	107	0.248
	114	27.0	24.5	181	0.315
	115	25.5	23.5	163	0.361
	116	26.5	24.0	167	0.262
	117	25.3	23.5	169	0.387
	118	25.1	23.3	166	0.299
	119	20.0	18.8	76	0.188
	120	19.3	17.6	65	0.121

Caldas Vizela	121	17.0	16.0	46	0.141
	122	17.2	16.2	52	0.210
	124	16.0	15.0	56	0.227
	125	18.2	16.8	65	0.219
	126	13.7	12.9	29	0.310
Ponte Trofa	136	16.0	14.7	35	0.159
	137	16.6	15.3	43	0.163
	138	13.9	12.7	29	0.040
	139	13.0	11.7	22	0.058
	140	12.5	11.4	18	0.070
Vizela Sto. Adrião	141	23.3	21.0	116	0.258
Prado	142	25.4	23.2	164	0.314
	143	20.4	19.9	88	0.217
	144	25.0	23.0	143	0.493
	145	24.1	22.0	112	0.298
	146	20.4	18.7	82	0.317

APPENDIX J

LPO and GST activity results for *Pseudochondrostoma* sp. (summer 2011). The results are expressed as mean ± standard error of the mean.

Location	LPO [nmol MDA/mg protein]	GST activity [nmol/min/mg protein]
Tejão	23.878 ± 10.858	36.563 ± 9.605
Pinhão	39.365 ± 29.764	16.020 ± 14.093
Prado	5.988 ± 1.053	45.205 ± 5.569
Vizela Sto. Adrião	27.130 ± 9.492	36.359 ± 6.207
Caldas Vizela	9.313 ± 2.616	78.467 ± 3.439

LPO and GST activity results for *Pseudochondrostoma* sp. (winter 2012). The results are expressed as mean ± standard error of the mean.

Location	LPO [nmol MDA/mg protein]	GST activity [nmol/min/mg protein]
Ponte Pingue	7.024 ± 1.254	36.820 ± 13.901
Prado	15.249 ± 6.152	50.633 ± 6.022
Caldas Vizela	9.458 ± 3.643	56.792 ± 10.569

APPENDIX K

LPO and GST activity for *L. bocagei* (summer 2011*).* The results are expressed as mean ± standard error of the mean.

Location	LPO [nmol MDA/mg protein]	GST activity [nmol/min/mg protein]
P. Junqueira	56.480 ± 18.830	10.981 ± 6.873
Vizela Sto. Adrião	24.790 ± 11.090	11.675 ± 2.461
Graça	14.990 ± 6.120	33.353 ± 10.940
Caldas Vizela	13.700 ± 6.130	47.474 ± 11.157

Lipid peroxidation, GST activity and SOD activity results for L. bocagei (winter 2012). The results are expressed as mean ± standard error of the mean (SEM).

Location	LPO [nmol MDA/mg protein]	GST activity [nmol/min/mg protein]	SOD activity [U SOD/mg protein]
P. Junqueira	12.470 ± 3.943	37.859 ± 5.404	1.630 ± 0.276
Trofa	15.520 ± 6.942	34.139 ± 12.642	0.960 ± 0.241
Prado	15.660 ± 7.004	22.497 ± 11.054	1.870 ± 0.369
Graça	5.490 ± 1.738	28.047 ± 5.847	1.810 ± 0.354
Caldas Vizela	5.380 ± 2.407	20.118 ± 4.701	1.260 ± 0.211