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War and Peace, Lev Tolstoy

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Publications

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Oliveira, P., Barboza, L.G.A., Branco, V., Figueiredo, N., Carvalho, C., Guilhermino, L. Effects of microplastics and mercury in the freshwater bivalve *Corbicula fluminea* (Müller, 1774): filtration rate, biochemical biomarkers and mercury bioconcentration. (Accepted in Ecotoxicology and Environmental Safety, corresponds to Chapter V, with permission of Elsevier included in the Annex I).

Oliveira, P., Guilhermino, L. Acclimation conditions for the use of the exotic invasive species *Corbicula fluminea* in toxicity bioassays. (In preparation, corresponds to Chapter II).

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Abstract

The protection of aquatic ecosystems and their resources is a priority of the European Union Water Framework Directive (WFD) (EC, 2000). To achieve a good ecological status, the reduction of the anthropogenic pressures exerted on water bodies, the prevention and mitigation of adverse effects due to global changes, including in relation to bioinvasions are of most importance.

Bioinvasions are considered a global problem because invasive species can change the structure and functioning of ecosystems and reduce biodiversity. The influence of anthropogenic pressures on the success of invasive species has been recognized, thus more investigation on the effects of environmental contaminants on exotic invasive species is essential for the establishment of plans for the prevention, management and control of bioinvasions.

Corbicula fluminea, commonly known as the Asiatic clam, is an exotic invasive freshwater bivalve species in Europe, United States of America and other regions. *C. fluminea* presents a strong invasive potential that allows the establishment of large populations in the invaded ecosystems, causing important ecological impacts and considerable economic losses. The species is used for human consumption in some areas in its native range.

The main objective of this Thesis was to investigate the effects of environmental contaminants on *C. fluminea*. Mercury was selected as a model contaminant because it is a priority hazardous substance under the WFD (EU, 2013), has a global distribution, long environmental persistence and is very toxic, posing a threat to animal, ecosystem and human health.

The specimens of *C. fluminea* used in this work were adult individuals collected in the Minho River upper estuary (Northwest Iberian Peninsula). This estuary was selected because it is included in the NATURA 2000 network, is considered a low impacted estuary and its *C. fluminea* population has been studied for several years. In the third study were also used adult specimens from the Lima River estuary.

A first study was carried out to determine the time period of acclimation to laboratorial conditions that should be used before using *C. fluminea* from wild populations in toxicity bioassays based on a set of sub-individual biomarkers. To achieve this objective, the activities of the enzymes cholinesterases (ChE), NADP-dependent isocitrate dehydrogenase (IDH), octopine dehydrogenase (ODH), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione *S*-transferases (GST), and the lipid

peroxidation levels (LPO) were determined immediately after arrival to laboratory, and after 7 and 14 days in controlled acclimation conditions. Bivalves were maintained in a room with a temperature of 16 ± 1 ºC and a photoperiod of 16 hours light/ 8 hours dark in tanks filled with dechlorinated tap water (hereafter indicated as clean medium). Changes of clean medium were carried out every 48 hours and bivalves were fed with a mixture of *Chlorella vulgaris* and *Chlamidomonas reinhardtii* (50%: 50% cells/cells) in a final concentration of 8×10^5 cells/mL/bivalve. After 7 days in such conditions, all biomarkers except ODH were significantly altered in relation to the corresponding levels determined immediately after arrival to the laboratory: LPO levels and the activity of the enzymes ChE, IDH and CAT were significantly increased, whereas GR, GPx and GST activities were significantly decreased. Such alterations indicate that after 7 days in the laboratory, bivalves were under stress. After 14 days of acclimation, all biomarkers returned to baseline levels determined immediately after arrival to the laboratory. Therefore, 14 days was found to be an adequate acclimation period before using *C. fluminea* from wild populations in toxicity bioassays using the tested biomarkers as effect criteria, and was selected as the acclimation period in the following experiments.

In a second study, the sensitivities of *C. fluminea* and of *Anodonta anatina* (native bivalve in Europe) to mercury were compared. After 14 days of acclimation in the conditions previously indicated (first study) individuals of the two species were independently exposed for 96 hours to mercury (31–500 µg/L) in laboratory semi-static conditions. No food was provided. The effect criteria were mortality and the biomarkers used in the first study. In the range of concentrations tested, 96 hours of exposure to mercury induced high mortality on *A. anatina* (up to 100% at 125 µg/L), whereas no mortality on *C. fluminea* was recorded. These results indicate that the native species was more sensitive to mercury than the invasive one, suggesting that the higher tolerance metal may beneficiate *C. fluminea* in scenarios of competition with *A. anatina* in mercury contaminated ecosystems. The biomarkers determined in *C. fluminea* indicated induction of defence mechanisms (up to 63 µg/L), and a significant ($p \leq 0.05$) and almost complete inhibition of IDH activity (96% at 500 µg/L) that could possibly be related with low oxygen levels resulting from long periods of valve closure, observed during the bioassay. Thus, valve closure and the effective activation of antioxidant defence mechanisms may have contributed to the relatively high tolerance of *C. fluminea* to mercury.

In the third study, the toxicity induced by 14 days of exposure to mercury on *C. fluminea* and the post-exposure recovery were investigated in relation to the potential influence of

environmental conditions of wild populations natural habitats. The approach consisted in comparing the responses of bivalves collected in the estuaries of Minho and Lima rivers. These ecosystems have several environmental differences, including in abiotic conditions and levels of some nutrients and contaminants, with the Lima River estuary being in general more contaminated than the Minho River estuary. Two independent semi-static bioassays were carried out simultaneously: one with bivalves from the Minho River estuary and the other with bivalves from the Lima River estuary. During the exposure period, bivalves were fed with a mixture of *Chlorella vulgaris* and *Chlamidomonas reinhardtii* (50%: 50% cells/cells) with a final concentration of 8×10^5 cells/mL/bivalve. The effect criteria were the following biomarkers: the oxygen consumption rate, the activities of ChE, IDH, ODH, CAT, GR, GPx and GST enzymes and the LPO levels. The biomarkers were determined in groups of animals at the end of the acclimation period and after the exposure to the following treatments: clean medium for 8 days; 31 µg/L of mercury for 8 days; 31 µg/L of mercury for 8 days followed by 6 days in clean medium (post-exposure recovery); clean medium for 14 days; and 31 µg/L of mercury for 14 days. For bivalves of both estuaries, no significant differences in any biomarker among the control groups were found. The integrated analysis of data (Three-way Analysis of Variance, fixed factors: estuary, time and mercury) indicated for several biomarkers: significant differences ($p \leq 0.05$) between bivalves from distinct estuaries; significant differences ($p \leq 0.05$) among animals exposed to distinct periods of time; significant differences ($p \leq 0.05$) between animals exposed to mercury and those not exposed to the metal; significant ($p \leq 0.05$) interaction between estuary and time; significant ($p \leq 0.05$) interaction between estuary and mercury; significant ($p \leq 0.05$) interaction between time and time mercury; and significant ($p \leq 0.05$) interaction among estuary, time and mercury. The further analyses of data indicated that after 8 days of exposure to mercury, bivalves from the Minho River estuary had significantly ($p \leq 0.05$) decreased GR activity while animals from the Lima River estuary had no alterations in any biomarker. The post-exposure recovery group of the Minho River estuary had significantly ($p \leq 0.05$) decreased oxygen consumption rate, inhibited IDH and GR activities and significantly increased LPO levels. No significant differences were found in animals from the Lima River estuary. Therefore, mercury induced delayed toxicity in bivalves from the Minho River estuary but not in those from the Lima River estuary. After 14 days of exposure to mercury, animals from both populations had significantly ($p \leq 0.05$) depressed oxygen consumption rate and IDH activity, suggesting changes in the cellular energy production pathways and reduced individual fitness. Moreover, at this period, decreased GR activity, increased GST activity and increased LPO levels were

observed in bivalves from Minho River estuary but not in those from Lima River estuary. Overall, the findings of this study indicated that: i) the exposure to 31 µg/L of mercury for 8 days and 14 days induced toxic effects on *C. fluminea*, ii) 6 days in clean medium was not sufficient to recover from 8 days of mercury exposure, a finding that has implications for human food safety, and iii) bivalves from the Minho River estuary were more sensitive to mercury exposure than those of the Lima River estuary.

Finally, a bioassay was carried out to investigate the combined effects of mercury and microplastics (another global pollutant of environmental, animal and human health concern) on *C. fluminea*. Bivalves were collected in the estuary of the Minho River estuary. The mercury body burden (whole soft body, hereafter indicated as body) was determined in a group of animals. The other bivalves were acclimated to laboratory conditions for 14 days (as previously described). At the end of that period, the body concentrations of mercury and the following biomarkers were determined in a group of animals: the activities of ChE, IDH, ODH, CAT, GR, GPx and GST and the LPO levels. The other bivalves were exposed to the following treatments: clean medium for 8 days; 0.13 mg/L of microplastics for 8 days; 0.03 mg/L of mercury for 8 days; mixture of microplastics (0.13 mg/L) and mercury (0.03 mg/L), hereafter indicated as mixture, for 8 days; clean medium for 14 days; 0.13 mg/L of microplastics for 8 days + clean medium for 6 days (post-exposure recovery); 0.03 mg/L of mercury for 8 days + clean medium for 6 days; and mixture for 8 days + clean medium for 6 days. Test medium was renewed every 24 hours, and animals were fed with a mixture (50%: 50% cells/cells) of *Chlorella vulgaris* and *Chlamidomonas reinhardtii,* in a final concentration of 8×10^5 cells/mL/bivalve. The concentrations of microplastics and mercury in test medium were determined at beginning, at the end and along the bioassay. After the exposure period, the concentrations of mercury in the body of animals and the biomarkers were determined. After 8 days, bivalves exposed to the metal alone and to the mixture had significantly ($p \leq 0.05$) increased body mercury concentrations. However the mercury bioconcentration was significantly lower in animals exposed to the mixture. After 8 days of exposure, mercury alone caused a significant ($p \leq 0.05$) decrease in the filtration rate (FR), in IDH, GR and GPx activities, as well as a significant increase in CAT and GST activities and in LPO levels. After 8 days of exposure to microplastics alone, particles were found in the digestive tract and in the gills. Moreover, animals exposed to microplastics alone had significant ($p \leq 0.05$) decreased FR, inhibited ChE, and increased LPO levels. After 8 days of exposure to the mixture, bivalves had significantly ($p \leq 0.05$) decreased FR, inhibited GR and GPx activities and increased CAT activity and LPO levels. Six days of post-exposure recovery in clean

medium was not sufficient for a complete recover of bivalves completely exposed to microplastics, mercury and mixture, since recovery was observed only in some biomarkers. Together, the results of this study indicate that microplastics influence the bioaccumulation and toxicity of mercury to *C. fluminea* and suggest antagonism between the two pollutants in this species.

Overall, the findings of the present Thesis provided a more in-depth view on the effects induced by mercury exposure in *C. fluminea*, on the mechanisms involved in the tolerance to mercury-induced stress and the post-exposure recovery capacity of this species. The knowledge of these aspects is intended to be a relevant contribution to a more effective management of *C. fluminea* bioinvasions and also to provide important data regarding public health by helping to establish or improve safety criteria for *C. fluminea* consumption.

Efeitos de contaminantes ambientais no bivalve exótico invasor *Corbicula fluminea* (Müller, 1774)

Resumo

A proteção dos ecossistemas aquáticos e dos seus recursos é uma prioridade da Diretiva-Quadro da Água da União Europeia (DQA) (EC, 2000). Para atingir um bom estado ecológico é fundamental a redução das pressões antropogénicas exercidas sobre as massas de água, bem como a prevenção e mitigação de efeitos adversos decorrentes das alterações globais, incluindo das bioinvasões.

As bioinvasões são consideradas um problema global, uma vez que as espécies invasoras podem alterar a estrutura e o funcionamento dos ecossistemas e reduzir a biodiversidade. A influência das pressões antropogénicas no sucesso das espécies invasoras tem vindo a ser reconhecida, pelo que o estudo dos efeitos de contaminantes ambientais nestas espécies é essencial para o estabelecimento de planos para a prevenção, gestão e controlo das bioinvasões.

Corbicula fluminea, também conhecida por amêijoa-asiática, é uma espécie de bivalve de água doce, exótica e invasora na Europa, Estados Unidos da América, entre outras regiões. *C. fluminea* apresenta um forte potencial invasor que permite o estabelecimento de grandes populações nos ecossistemas invadidos, provocando impactos ecológicos importantes e prejuízos económicos consideráveis. *C. fluminea* é utilizada para consumo humano em algumas regiões onde a espécie é nativa.

A presente Tese teve como objetivo principal investigar os efeitos de contaminantes ambientais em *C. fluminea*. O mercúrio foi selecionado como contaminante modelo porque é uma substância perigosa prioritária no âmbito da DQA (EU, 2013), tem uma distribuição global, elevada persistência ambiental e apresenta uma elevada toxicidade, constituindo, assim, uma ameaça à saúde ambiental, animal e humana.

Os espécimes de *C. fluminea* utilizados nos trabalhos a seguir apresentados foram recolhidos no seu estado adulto na parte superior do estuário do Rio Minho (Noroeste da Península Ibérica). Este estuário foi selecionado porque está incluído na Rede NATURA 2000, é considerado um estuário com baixo nível de pressão antropogénica, e porque a população de *C. fluminea* tem vindo a ser investigada há vários anos. Num dos estudos

foram também utilizados espécimes adultos de *C.fluminea* da população do estuário do Rio Lima.

O primeiro estudo teve como objetivo a determinação do período de aclimatação laboratorial adequado para a utilização de *C. fluminea* proveniente de populações selvagens em bioensaios de toxicidade baseados num conjunto de biomarcadores sub-individuais. Imediatamente após a chegada ao laboratório, e após 7 e 14 dias em condições laboratoriais controladas, foram determinadas as atividades das enzimas colinesterases (ChE), isocitrato desidrogenase dependente de NADP (IDH), octopina desidrogenase (ODH), catalase (CAT), glutationa redutase (GR), glutationa peroxidase (GPx) e glutationa *S*-transferases (GST) e os níveis de peroxidação lipídica (LPO). Os bivalves foram mantidos numa sala com temperatura de 16 ± 1 ºC e fotoperíodo de 16 horas de luz/8 horas de escuridão em tanques com água da torneira desclorada (doravante designada por meio limpo). As mudanças de meio limpo foram realizadas a cada 48 horas e os bivalves foram alimentados com uma mistura de *Chlorella vulgaris* e *Chlamidomonas reinhardtii* (50%: 50% células/células) numa concentração final de 8×10^5 células/mL/bivalve. Após 7 dias nestas condições, todos os biomarcadores, exceto a ODH, encontravam-se significativamente alterados em relação aos níveis correspondentes determinados imediatamente após a chegada ao laboratório: os níveis de LPO e a atividade das enzimas ChE, IDH e CAT encontravam-se significativamente aumentados, enquanto as actividades da GR, GPx e GST encontravam-se significativamente diminuídas. Estas alterações sugerem que após 7 dias no laboratório os bivalves encontravam-se sob *stress*. Após 14 dias de aclimatação às condições laboratoriais definidas, todos os biomarcadores regressaram aos níveis basais determinados imediatamente após a chegada ao laboratório. Concuiu-se, assim, que 14 dias é o período de aclimatação adequado para a utilização de *C. fluminea* proveniente de populações selvagens em bioensaios de toxicidade que utilizem como critérios de efeito os biomarcadores testados neste trabalho. Por esse motivo, foi também definido como o período de aclimatação dos bioensaios a seguir apresentados.

No segundo estudo foi comparada a sensibilidade de *C. fluminea* e de *Anodonta anatina* (bivalve nativo na Europa) ao mercúrio. Após 14 dias de aclimatação às condições previamente indicadas (primeiro estudo), os indivíduos das duas espécies foram expostos independentemente durante 96 horas a mercúrio (31–500 µg/L) em condições laboratoriais semi-estáticas. Não foi fornecido qualquer alimento no decorrer do ensaio. A taxa de mortalidade e os biomarcadores utilizados no primeiro estudo foram utilizados como critérios de efeito. No intervalo de concentrações testadas, a exposição ao mercúrio durante 96 horas

induziu uma elevada mortalidade em *A. anatina* (100% nos bivalves expostos a 125 µg/L), enquanto não foi registada qualquer mortalidade em *C. fluminea*. Estes resultados indicam uma maior sensibilidade ao mercúrio da espécie nativa comparativamente à espécie invasora, sugerindo que a tolerância mais elevada de *C. fluminea* poderá, eventualmente, beneficiá-la em cenários de competição com *A. anatina* em ecossistemas contaminados por mercúrio. Os biomarcadores determinados em *C. fluminea* indicaram a indução de mecanismos de defesa (até 63 µg/L) e a diminuição significativa ($p \leq 0.05$) da atividade da IDH (96% nos bivalves expostos a 500 µg/L). Esta inibição poderá estar relacionada com baixos níveis de oxigénio resultantes de longos períodos de fechamento das valvas observados no decorrer do bioensaio. Assim, o fechamento das valvas e a ativação efetiva de mecanismos de defesa antioxidante parecem estar na base da tolerância relativamente elevada de *C. fluminea* ao mercúrio.

No terceiro estudo, foi investigada a toxicidade induzida pela exposição ao mercúrio durante 14 dias e a recuperação pós-exposição de *C. fluminea* em relação à potencial influência das condições ambientais dos habitats naturais de duas populações selvagens. A abordagem consistiu na comparação das respostas de bivalves provenientes das populações dos estuários dos rios Minho e Lima. Estes ecossistemas apresentam várias diferenças ambientais, incluindo nas condições abióticas e nos níveis de alguns nutrientes e contaminantes, sendo o estuário do Rio Lima, em geral, mais contaminado do que o estuário do Rio Minho. Foram realizados em simultâneo dois bioensaios independentes, em condições semi-estáticas: um com bivalves do estuário do Rio Minho e outro com bivalves do estuário do Rio Lima. Durante o período de exposição, os animais foram alimentados com uma mistura de *Chlorella vulgaris* e *Chlamidomonas reinhardtii* (50%: 50% células/células) numa concentração final de 8×10^5 células/mL/bivalve. Os critérios de efeito foram os seguintes biomarcadores: a taxa de consumo de oxigénio; as actividades das enzimas ChE, IDH, ODH, CAT, GR, GPx e GST; e os níveis de LPO. Os biomarcadores foram determinados em grupos de animais após o período de aclimatação e após a exposição aos seguintes tratamentos: meio limpo durante 8 dias; 31 µg/L de mercúrio durante 8 dias; 31 µg/L de mercúrio durante 8 dias, seguidos de 6 dias em meio limpo (recuperação pós-exposição); meio limpo durante 14 dias; e 31 µg/L de mercúrio durante 14 dias. Não foram encontradas diferenças significativas em qualquer biomarcador entre os grupos controlo dos bivalves dos estuários dos rios Minho e Lima. A análise integrada dos dados (Análise de Variância de três fatores; fatores fixos: estuário, tempo e mercúrio) indicou para vários biomarcadores: diferenças significativas ($p \leq 0.05$) entre bivalves dos dois estuários;

diferenças significativas ($p \leq 0.05$) entre animais expostos durante distintos períodos de tempo; diferenças significativas ($p \leq 0.05$) entre animais expostos ao mercúrio e aqueles não expostos ao metal; interação significativa ($p \leq 0.05$) entre estuário e tempo; interação significativa ($p \leq 0,05$) entre estuário e mercúrio; interação significativa ($p \leq 0,05$) entre tempo e mercúrio; e interação significativa ($p \leq 0.05$) entre estuário, tempo e mercúrio. As análises posteriores mostraram uma diminuição significativa ($p \leq 0.05$) da atividade da GR nos bivalves do estuário do Rio Minho após 8 dias de exposição ao mercúrio, enquanto os animais do estuário do Rio Lima não apresentaram quaisquer alterações. O grupo de recuperação pós-exposição do estuário do estuário do Rio Minho apresentou uma diminuição significativa ($p \leq 0.05$) da taxa de consumo de oxigénio, das actividades da IDH e da GR e um aumento significativo dos níveis de LPO. Nos bivalves do estuário do rio Lima não foi encontrada qualquer diferença significativa, concluindo-se, assim, que o mercúrio induziu toxicidade retardada nos bivalves do estuário do Rio Minho, mas não nos animais do estuário do Rio Lima. Após 14 dias de exposição ao mercúrio, os animais de ambas as populações apresentaram uma diminuição significativa ($p \leq 0.05$) da taxa de consumo de oxigénio e inibição da atividade da IDH, resultado que sugere alterações nas vias celulares de produção de energia e uma redução do estado geral de saúde individual. Além disso, neste período os bivalves do estuário do Rio Minho apresentaram a atividade da GR significativamente inibida e a atividade da GST e os níveis de LPO significativamente aumentados, o que não se verificou nos bivalves do estuário do Rio Lima. Em conclusão, os resultados deste estudo indicaram que: i) a exposição a 31 µg/L de mercúrio durante 8 e 14 dias induziu efeitos tóxicos em *C. fluminea*, ii) um período de 6 dias em meio limpo não foi suficiente para recuperar da exposição ao mercúrio durante 8 dias (um dado que tem implicações para a segurança alimentar humana) e iii) os bivalves do estuário do Rio Minho são mais sensíveis à exposição ao mercúrio do que os do estuário do Rio Lima.

Por último, foi realizado um bioensaio com o objetivo de investigar os efeitos combinados de mercúrio e microplásticos (outro poluente global preocupante a nível da saúde ambiental, animal e humana) em *C. fluminea.* Os bivalves foram recolhidos na parte superior do estuário do Rio Minho. A concentração de mercúrio no corpo total de *C. fluminea* (corpo mole inteiro, doravante designado por corpo) foi determinada num grupo de animais. Os restantes bivalves foram aclimatados durante 14 dias às condições laboratoriais descritas anteriormente. No fim desse período, as concentrações corporais de mercúrio e os seguintes biomarcadores foram determinados num grupo de animais: atividades das enzimas ChE, IDH, ODH, CAT, GR, GPx e GST e os níveis de LPO. Os restantes bivalves foram expostos

aos seguintes tratamentos: meio limpo durante 8 dias; 0.13 mg/L de microplásticos durante 8 dias; 0.03 mg/L de mercúrio durante 8 dias; mistura de microplásticos (0.13 mg/L) e mercúrio (0.03 mg/L) durante 8 dias, a seguir indicada como mistura; meio limpo durante 14 dias; 0.13 mg/L de microplásticos durante 8 dias + meio limpo durante 6 dias (recuperação pós-exposição); 0.03 mg/L de mercúrio durante 8 dias + meio limpo durante 6 dias e mistura durante 8 dias + meio limpo durante 6 dias. Os meios de teste foram renovados a cada 24 horas e os animais foram alimentados com uma mistura (50%: 50% células/células) de *Chlorella vulgaris* e *Chlamidomonas reinhardtii*, numa concentração final de 8×10^5 células/ml/bivalve. As concentrações de mercúrio e microplásticos nos meios de teste foram determinadas no início, no fim e ao longo do bioensaio. Após o período de exposição foram determinadas as concentrações de mercúrio no corpo dos animais e os biomarcadores. Os bivalves expostos apenas ao metal e à mistura apresentaram concentrações de mercúrio significativamente ($p \leq 0.05$) aumentadas. Contudo, a bioconcentração de mercúrio foi significativamente inferior nos animais expostos à mistura. Após 8 dias de exposição ao mercúrio verificou-se uma redução significativa ($p \leq 0.05$) na taxa de filtração, nas atividades da IDH, GR e GPx, bem como um aumento significativo das atividades da CAT e da GST e dos níveis de LPO. Após 8 dias de exposição a microplásticos, foi detetada a presença de partículas no trato digestivo e nas brânquias. Além disso, os animais expostos a este tratamento apresentaram uma diminuição significativa ($p \leq 0.05$) da taxa de filtração e da atividade da ChE e um aumento significativo dos níveis de LPO. Após 8 dias de exposição à mistura, foi observada uma diminuição significativa ($p \leq 0.05$) da taxa de filtração, das atividades da GR e da GPx e um aumento significativo da atividade da CAT e dos níveis de LPO. O período de 6 dias em meio limpo revelou-se insuficiente para a recuperação completa dos bivalves às exposições a microplásticos, mercúrio e mistura, uma vez que se observou recuperação apenas em alguns biomarcadores. Em conjunto, os resultados deste estudo indicam que os microplásticos influenciaram a bioacumulação e a toxicidade do mercúrio em *C. fluminea* e sugerem antagonismo entre os dois poluentes nesta espécie.

No geral, os resultados da presente Tese apresentam uma visão mais aprofundada sobre os efeitos induzidos pela exposição ao mercúrio em *C. fluminea,* sobre os mecanismos envolvidos na tolerância ao *stress* induzido pelo metal e sobre a capacidade de recuperação da espécie. Pretende-se que o conhecimento destes aspetos seja um contributo relevante para uma gestão mais eficiente das bioinvasões de *C. fluminea*, e que possa também fornecer dados importantes para a saúde pública, tendo em vista o melhoramento ou o estabelecimento de critérios de segurança para o consumo desta espécie.

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List of abbreviations

ANOVA - Analysis of Variance

APA - Agência Portuguesa do Ambiente

BCF - Bioconcentration factor

CAT - Catalase enzyme

CDNB - 1-Chloro-2,4-dinitrobenzene

ChE - Cholinesterase enzymes

DO - Dissolved oxygen

DTT - DL-1,4-Dithiothreitol

Dw - Dry weight

EC - European Commission

EDTA - Ethylenediaminetetraacetic acid

EU - European Union

GSH - Glutathione (reduced form)

GSSG - Glutathione (oxidized form)

GPx - Glutathione peroxidase enzyme

GR - Glutathione reductase enzyme

GST - Glutathione *S*-transferases enzymes

IDH - isocitrate dehydrogenase enzyme

LC_{50} - Median lethal concentration: the concentration of the tested substance estimated to cause 50% of mortality in the tested population in the specific conditions of the toxicity bioassay.

LC_{20} - 20% lethal concentration: the concentration of the tested substance estimated to cause 20 % of mortality in the tested population in the specific conditions of the toxicity bioassay.

LC_{10} - 10% lethal concentration: the concentration of the tested substance estimated to cause 20% of mortality in the tested population in the specific conditions of the toxicity bioassay.

LT_{50} - Median lethal time: the estimated time (hours) necessary to induce 50% of mortality in the tested population under exposure to a certain concentration of the tested substance in the specific conditions of the toxicity bioassay.

L-est - Lima River estuary

LPO - Lipid peroxidation

M-est - Minho River estuary

MP - Microplastics

MT - Metallothionein

MXR - Multixenobiotic resistance

NAD^+ - Nicotinamide adenine dinucleotide (oxidized form)

NADH Nicotinamide adenine dinucleotide (reduced form)

$NADP^+$ - Nicotinamide adenine dinucleotide phosphate (oxidized form)

NADPH - Nicotinamide adenine dinucleotide phosphate (reduced form)

NIS - Non-indigenous species

NW - Northwest

OD - Optical density

ODH - Octopine dehydrogenase enzyme

OECD - Organisation for Economic Co-operation and Development

OPSPAR Convention - Oslo and Paris Convention for the Protection of the Marine Environment of the North-East Atlantic

Ppm - Parts per million

Psu - Practical salinity units

ROS - Reactive oxygen species

RFU - Relative fluorescence units

SD - Standard deviation

S.E.M - Standard error of the mean

SOD - Superoxide dismutase enzyme

TBARS - Thiobarbituric acid reactive substances

UNEP - United Nations Environment Programme

WFD - Water Framework Directive

CHAPTER I

General Introduction

1.1. Bioinvasions

"The real thing is that we are living in a period of the world's history when the mingling of thousands of kinds of organisms from different parts of the world is setting up terrific dislocations in nature" (Elton, 1958)

Freshwater and estuarine ecosystems have been continuously subjected to critical threats including habitat destruction, climate changes, pollution and bioinvasions (Meybeck, 2003). Bioinvasions are one of the most significant problems to ecosystem integrity and biodiversity (Sousa *et al*., 2011; Gangloff *et al*., 2016; O'Brien *et al*., 2016). Although bioinvasions are part of Earth's evolutionary processes, they are now a global paradigm with implications to environmental, animal and human health, mainly because they are occurring at unprecedented rates (Ricciardi, 2007; Simberloff *et al*., 2013; Ochocki and Miller, 2017). Since the publication of the monograph "The Ecology of Invasions by Animals and Plants" by Charles Elton in 1958 (Elton, 1958) and the emergence of invasion ecology as a discipline, the anthropogenic dimension of bioinvasions has been unquestionably recognized (Pyšek and Richardson, 2010). The development of a wide range of diverse human activities has led to the emergence of new routes allowing the introduction of several species into territories where these species did not exist before. The globalization of trade has been pointed as the principal driver of bioinvasions in aquatic systems (Levine and D'Antonio, 2003; Karatayev *et al*., 2007; Hulme, 2009). Moreover, climate changes have the potential to increase the likelihood of expansion of some invasive species beyond their native distribution (Hulme, 2017).

One of the proposed frameworks for bioinvasions divides the process into four main stages: transport, introduction, establishment and spread (Blackburn *et al*., 2011). The first step is the overcoming of an obstacle that prevents the movement of the species. This can occur without assistance, when the species has natural dispersal ability, but sometimes it can happen through human-mediated transport, whether in accidental or deliberate ways. Vectors and pathways through which exotic species can be introduced in a new area include ballast water, aquaculture, fish baits, aquarium and ornamental trades, tourism and recreational activities (Padilla and Williams, 2004; Gollasch, 2006; Williams *et al*., 2013; Patoka *et al.,* 2017; Rhyne *et al*., 2017). The introduction *per se* happens with the arrival of the species at the new location. After a successful establishment, the dispersion may occur to a greater or lesser extent, depending on a combination of the characteristics of the recipient ecosystem and the

species itself (Chapple *et al*., 2012). Measures to combat invasive species can be applied at any stage of bioinvasions but their effectiveness will be higher if implemented at early stages of introduction and establishment phases, whereas their efficacy tend to decrease and costs tend to increase at later stages of establishment and spread (Epanchin-Niell, 2017).

A species introduced into a new environment is referred as non-native, non-indigenous, exotic or alien, and when it establishes and spreads very rapidly is also called invasive (Colautti and MacIsaac, 2004). The introduction and spread of non-indigenous species (NIS) in terrestrial and aquatic habitats is well documented and became a topic of special concern in the field of Ecology. The impacts of NIS assume variable forms and can affect the recipient biota at different organizational levels (Strayer, 2010; Ricciardi *et al*., 2013). The ecological impacts of NIS in the abundance and diversity of existing communities can be direct (*e.g.* predator-prey interactions, parasitism, hybridization or diseases) or indirect, when the NIS alters the habitat structure or interferes with trophic webs and energy fluxes (Crooks, 2002; Schmidlin and Baur, 2007; Gallardo *et al*., 2016). Moreover, NIS can threat human health by acting as potential vectors of diseases (Conn, 2014) and also cause considerable economic losses. The annual economic costs associated with damage and control of NIS are estimated to be ~120 billion $ in the U.S.A and 12.5 billion € in Europe (Pimentel *et al*., 2005; Kettunen *et al*. 2008). According to the project “Delivering Alien Invasive Species Inventory for Europe” (DAISIE, 2018), the number of NIS successfully established in Europe has been increasing exponentially, currently reaching more than 12000. From these, around 15% are adversely affecting the biodiversity and causing losses of billions of Euros every year (Hulme *et al*., 2009; Latombe *et al*., 2016). In these regards, the monitoring of NIS is one of the descriptors of the European Union (EU) Marine Strategy Framework Directive (EC, 2008a) that aims to achieve or maintain a “Good Environmental Status” of the EU marine waters by 2020. Moreover, the European Parliament adopted the EU Biodiversity Strategy to 2020 (EC, 2011) whose Target 5 is to combat NIS by minimizing their negative impacts on biodiversity through measures that include an early detection and eradication of recently arrived NIS, and the effective management of those already established. As part of the Convention on Biological Diversity, the EU provided the legal framework to combat NIS, through the Regulation on the “Prevention and Management of the Introduction and Spread of Invasive Alien Species” (EU, 2014). This regulation aims the protection of the biodiversity, ecosystem services and human health and establishes that the detection, early eradication and management must be carried out by Member States. More recently, the European Commission (EC) also adopted the first

list of “Invasive Alien Species of Union concern” (EU 2016; EU, 2017). This list includes all the species subjected to restrictions of keeping, importing, selling, breeding and growing.

Aquatic bioinvasions are of special concern because the current and future extinction rates are estimated to be five times higher than those occurring in terrestrial environments (Ricciardi *et al*., 1998). Moreover, due to intense anthropogenic pressures and a high number of dispersal vectors, freshwaters and transitional waters are considered particularly susceptible to bioinvasions (Ricciardi and Kipp, 2008). Among aquatic faunal groups, bivalve molluscs stand out for their ability to disrupt trophic chains, alter nutrient fluxes and control the structure and functioning of the invaded ecosystems (Vaughn and Hakenkamp, 2001). Furthermore, there is evidence of a relationship between the introduction of bivalve NIS and declines of the native ones (Ricciardi *et al*., 1998; Ricciardi and Whoriskey, 2004). In view of these considerations, one of the main goals of invasion biology is to identify the factors that influence the likelihood of bioinvasions and the success of NIS in the recipient ecosystems (Kolar and Lodge, 2001; Walther *et al*., 2009; Mächler and Altermatt, 2012), which is crucial to define and implement strategies to prevent or manage the impacts of these species (Epanchin-Niell, 2017).

In general, bionvasions are known to occur more frequently in human-altered habitats (Dafforn *et al*., 2009; Sullivan *et al*., 2015), and a positive correlation between the invasiveness and the degree of disturbance has been established (Preisler *et al*. 2009; Tamburello *et al*., 2014; Bulleri *et al*., 2016). Anthropogenic pressures can lead to a reduction of habitat quality, affecting the lifecycle and health *status* of resident species (Bogan, 1993). Several human-generated disturbances such as regularization of rivers by man-made structures, draining activities and pollution facilitate the invasion and establishment of NIS (Lozon and MacIsaac, 1997; Salomidi *et al*., 2013). Chemical contamination from anthropogenic sources is a particularly important form of disturbance of aquatic environments that may favor the success of NIS in different ways and at different stages of bioinvasions (McKenzie *et al*., 2012). Environmental contaminants may favour the introduction of NIS because they can cause significant degradation of habitats, negatively affecting native species (Crooks *et al*., 2011). Additionally, NIS often present characteristics that represent advantages over their native competitors (Piola and Johnston 2008, 2009). In fact, tolerance to environmental contamination is pointed out as one of the factors contributing to the success of NIS over native species in aquatic ecosystems (Bielen *et al*., 2016). However, the results of several studies comparing tolerances of invasive species and native taxonomically related ones are contradictory (Prenter *et al*., 2004; Faria *et al*., 2010; Lenz *et al*., 2011; Velez

et al., 2016), showing that invasive species are not always the most tolerant. Thus, this topic requires further investigation.

1.2. *Corbicula fluminea* (Müller, 1774)

Corbicula fluminea (Müller, 1774) (Bivalvia: Corbiculidae), also known as the Asiatic clam, is a native species to Asia, Africa and Australia that has been spreading to multiple ecosystems all over the world (Mouthon 1981; Counts 1986; Araujo *et al*., 1993; Lucy *et al*., 2012; Crespo *et al*., 2015). It has a marked invasive behaviour and is included in the list of the 100 worst NIS in Europe (DAISIE, 2018). The first record of *C. fluiminea* outside its native range was in 1924 in Vancouver Island, British Columbia, Canada (Mcmahon, 1983). The first introduction of *C. fluminea* in South America occurred in Argentina around 1960s - 1970s (Ituarte, 1981) and since then its presence has been reported in Uruguay, Paraguay and Southern Brazil (Cataldo and Boltovskoy, 1998). In late 1970s - early 1980s, *C. fluminea* was introduced in Europe, being reported for the first time in France and Portugal by Mouthon (1981).

1.2.1. Biology and ecology of *C. fluminea*

C. fluminea is generally described as a hermaphroditic species, which reproduction occurs mainly through cross-fertilization (Rajagopal *et al*., 2000; Park and Chung, 2004) but self-fertilization can also be observed (Kraemer *et al*., 1986). The reproduction is initiated by favourable environmental conditions, especially increased water temperatures and high food availability (Doherty *et al*., 1987; Mouthon, 2001; Beekey and Karlson, 2003). Although depending on the ecosystem, *C. fluminea* usually presents a bivoltine reproductive cycle, with two spawning periods: one between the the late spring and the early summer and the other between the late summer and the autumn. (Rajagopal *et al*. 2000; Mouthon and Parghentanian 2004; Sousa *et al*., 2008a). Nevertheless, some studies report an almost continuous reproduction with no clear patterns of gamete release and spawning (Oliveira, 2015; Cao *et al*., 2017). The fertilization occurs inside the paleal cavity of adult individuals, and the fertilized eggs and pediveliger larvae are kept in the inner demibranch (Kraemer and Galloway, 1986). The incubation period depends on environmental conditions, varying between 6 to 60 days, usually taking two weeks (King *et al*. 1986, McMahon 1999). After veliger and pediveliger stages, the larvae of *C. fluminea* have a “D” shape configuration with straight hinged shells measuring about 250 µm (anterior-posterior length) (King *et al*., 1986;

McMahon, 1999). At this stage, larvae are released from the gills' chambers via the exhalant siphon into the surrounding water, and after four days they settle in the sediment (Araujo *et al*. 1993; McMahon, 1999). Under favourable hydrological conditions, they can be released from sediments back to the water column (McMahon, 1999). The sexual maturation is reached between 3 to 6 months of age (McMahon, 1999). The lifespan of *C. fluminea* is variable but usually ranges from 1 to 5 years (Sousa *et al*. 2008a). In the adult stage, the anterior-posterior length of the shell is, in average, 30 mm (McMahon, 2002) (Fig. 1).

Fig. 1. *Corbicula fluminea* with visible inhalant and exhalant siphons.

The high metabolic rates and rapid growth of *C. fluminea* are due, in part, to its feeding strategy (Hakenkamp and Palmer 1999). It feeds mainly on phytoplankton and bacteria present in the water column through water filtration (Beaver *et al*., 1991; Boltovskoy *et al*., 1995) but when planktonic food is not abundant it can also assimilate organic matter from the sediment using the foot, a mechanism designated as pedal feeding (Hakenkamp and Palmer, 1999). Pedal feeding is the primary feeding mechanism in larvae until the development of filtration structures is complete (McMahon, 1991; Reid *et al*., 1992; Hakenkamp *et al*., 2001).
C. fluminea is a freshwater bivalve species (McMahon, 1999) that tolerates salinity levels up to 17 psu (Britton and Morton, 1982; Franco *et al*., 2012; Verbrugge *et al.*, 2012; Modesto *et al*., 2013; Crespo *et al*., 2017). This indicates a good adaptation to brackish conditions, which is possibly related to efficient osmoregulation mechanisms (Morton and Tong, 1985, McMahon, 1991). This tolerance allows the species to colonize downstream estuarine areas (Sousa *et al*., 2008b; Franco *et al*., 2012; Ilarri *et al*., 2014).

C. fluminea occurs both in lentic and lotic habitats (Britton and Morton, 1982) showing preference for well oxygenated sediments containing high levels of organic matter, such as mixtures of sand with silt and clay, but it can also be found in other types of substrates (Belanger *et al*., 1985; Hakenkamp and Palmer 1999; Vaughn and Hakenkamp 2001).

C. fluminea has a wide thermal tolerance (2 to 37 °C) (McMahon and Williams; 1986; Müller and Baur, 2011; Rosa *et al*., 2012) surviving in the lower and upper limits of temperature for at least short periods of time. Nevertheless, the growth and reproduction seem to be compromised at water temperatures below 10 °C (Britton and Morton, 1979; Karatayev *et al*., 2005). Thus, water temperature seems to limit the dispersion of the species (Rosa *et al*., 2012). Notwithstanding, some future climatic scenarios suggest that further expansion of *C. fluminea* into higher latitudes is likely to occur (Crespo *et al*, 2015; Gama *et al*., 2017).

C. fluminea is low tolerant to water pH levels below 5 units, and calcium concentrations lower than 3 mg/L (Mackie and Claudi, 2010; Ferreira-Rodríguez *et al*., 2017).

The known natural predators of *C. fluminea* in European and North American ecosystems include fish (*e.g. Barbus* spp*., Luciobarbus* spp., *Cyprinus carpio* and *Lepomis gibbosus*) and invertebrates (*e.g. Procambarus clarkii*) that consume the smaller specimens (Pereira *et al*., 2016).

1.2.2. *C. fluminea* bioinvasions

The introduction of *C. fluminea* into new areas is closely related to human activities, including transport of individuals in ballast ship waters, displacement of specimens by tourists, use as fish bait, aquarium releases and trade as food items (McMahon, 1999; 2002; Darrigran, 2002). The high dispersion capacity of the species is partly due to the fact that the released larvae are completely formed and, although they do not actively swim, they can float and be drawn by the currents to long distances downstream (Prezant and Chalermwat, 1984). Besides that, the secretion of a mucilaginous drogue line observed in juveniles and adults assists floatation and promotes the drift to new locations (Prezant and Chalermwat, 1984; Rosa *et al*., 2014a). Pediveliger larvae are adapted to crawl (Britton and Morton, 1982), which allows moving upstream in slow waters (Voelz *et al*., 1998). Some external biotic vectors can also assist the spread of the species; juveniles and adults can be transported to distant locations in feet and feathers of birds, which may be important for secondary introductions (Green and Figuerola, 2005).

In the last four decades, *C. fluminea* has been spreading to several European countries, from Portugal to Romania (Minchin, 2014), including the British Islands (Elliott and Ermgassen, 2008; Lucy *et al*., 2012).

The first record of *C. fluminea* in Portugal was in the Tejo River estuary in 1978 (Mouthon, 1981). It is likely that the species has spread from this system to other continental freshwater ecosystems through human activities and/or natural dispersion by other organisms (Gomes *et al*., 2016). Nowadays, *C. fluminea* can be found in the most important Portuguese river basins, including those of Minho, Lima, Douro, Mondego, Sado and Guadiana Rivers (Chainho *et al*., 2006; Sousa *et al*., 2006a, 2008d; Morais *et al*., 200; Rosa *et al*., 2011) and in sites where until recently there were no records of its presence, such as the Cávado, Ave and Leça Rivers (Rosa *et al*., 2011; APA, 2014). In the Minho River estuary (M-est), *C. fluminea* plays a particularly important role because it constitutes more than 90% of the benthic faunal biomass (Sousa *et al*. 2008b, 2008c, 2008e; Ferreira-Rodríguez and Pardo, 2016). Since its introduction, known in 1989 (Araujo *et al*., 1993), the species has dispersed and thrived along the estuary. For this reason*, C. fluminea* inhabiting the M-est has been extensively studied in relation to several aspects including its ecology and invasive behaviour, impacts on native species, and population heath status (Sousa *et al*., 2008c, 2008f; Ilarri *et al*., 2012; Oliveira *et al*., 2015a; Novais *et al*., 2016). *C. fluminea* is also present in the Lima River estuary (L-est) that is located near the M-est, and although they share some similar hydromorphological characteristics, they have also important differences regarding abiotic conditions and anthropogenic pressures, including in the levels of several environmental contaminants (Costa-Dias *et al*., 2010; Guimarães *et al*., 2012; Rodrigues *et al*., 2014; Baeta *et al*., 2017). In the L-est, *C. fluminea* was recorded for the first time in 2002, and has a lower density and a more sparse distribution than in the M-est (Sousa *et al*., 2006a, 2006b; Ilarri *et al*., 2011).

1.2.3. Factors influencing the invasive behaviour of *C. fluminea*

The remarkable life strategies of *C. fluminea* coupled with high physiological and ecological plasticity (Dybdhal and Kane, 2005) allows the species to quickly adapt to the invaded ecosystems, reaching high densities and often becoming the dominant benthic species few years after its introduction (Katarayev *et al*., 2003; Elliot and Ermgassen, 2008; Werner and Rothhaupt, 2007; Sousa *et al*, 2008d). Its high invasive capacity is related to particular traits, mostly associated with a *r* – strategy, including high fecundity, early sexual maturation, high growth rates and short life-span (Aldridge and McMahon 1978; McMahon, 2002).

Hermaphroditism and self-fertilization are also pointed as important factors for the invasive success of the species (Pigneur *et al*., 2012), as a single individual can give rise to an offspring of 90 000 in just one reproductive period (McMahon, 1999). *C. fluminea* has higher filtration rates than most bivalves, as well as high metabolic and assimilation rates and consequently rapid growth (McMahon 1999). These characteristics allow the establishment of large populations in short periods of time. A relatively high tolerance to several chemical contaminants (Doherty, 1990; Guo and Feng, 2018) may also play an important role in its invasive success.

1.2.4. Impacts of *C. fluminea*

The ecological relevance of *C. fluminea* is based on its ability to change complex physicochemical processes in the water column and in the sediment-water surface (Sousa *et al*., 2008d; Sousa *et al*., 2009; Bullard and Hershey, 2013). *C. fluminea* is thus considered to play a key role as an ecosystem engineer (Sousa *et al*., 2009). The combination of feeding strategies, biodeposition and bioturbation activities affects the nutrient cycles, oxygen availability and sedimentation rates of the invaded ecosystems, impacting both benthic and pelagic species (Hakenkamp and Palmer, 1999; Werner and Rothhaupt, 2007; Sampaio and Rodil, 2014). Since *C. fluminea* has high filtration and assimilation rates (Way *et al*. 1990; Silverman *et al*. 1995) it may advantageously compete for food with native bivalves by reducing phytoplankton availability (Cohen *et al*. 1984; Boltovskoy *et al*., 1995; McMahon, 1999; Vaughn and Hakenkamp, 2001; Kamburska *et al*., 2013). Additionally, because *C. fluminea* is a non-selective feeder the overlap of diets can also occur (Atkinson *et al*., 2011). *C. fluminea* has a negative impact on the recruitment of native species by ingesting large amount of sperm, larvae or newly metamorphosed juveniles (Strayer, 1999; Yeager *et al*., 1999). Bioturbation, burrowing, pedal feeding activities may also reduce or destroy the habitat available for juvenile unionids (Hakenkamp and Palmer, 1999; Yeager *et al*., 1999).

Through pedal feeding *C. fluminea* reduces the organic matter content of sediments and the amount of benthic bacteria and diatoms (Hakenkamp *et al*. 2001). *C. fluminea* can destroy the superficial layers of sediment, affecting porosity, permeability and grain size, increasing oxygen penetration and water content and enhancing microbial activity (Zhang *et al*., 2011).

Due to high excretion rates, *C. fluminea* biodeposites large amounts of faeces and pseudofaeces, leading to the release of large amounts of ammonia and phosphates. Thus, it shows a great ability to interfere in nutrient cycling (Lauritsen and Mozley 1989; Vaughn and Hakenkamp, 2001; Xiao *et al*., 2014) and those impacts could be magnified in climate change

scenarios such as droughts and heat waves, as recently demonstrated by Coelho *et al*. (2018).

The exposure to extreme conditions such as prolonged drought, high temperatures, low oxygen levels and low redox potential, among others, can result in massive mortality of *C. fluminea* (Ilarri *et al*., 2011; Oliveira *et al*., 2015a; McDowell *et al*., 2017). Bivalves, in general can undergo periodic mass mortality events, but *C. fluminea* seems to recover faster than their native competitors (Sousa *et al*., 2008c, Sousa *et al*., 2012). Moreover, the organic matter resulting from the decomposition of dead individuals can lead to an overload of nutrients affecting the benthic fauna and deteriorating the water quality (Cherry *et al*., 2005; Cooper *et al*., 2005; Schmidlin and Baur, 2007; Werner and Rothhaupt, 2007).

The strong invasive character of *C. fluminea* is associated with a variety of competitive advantages over native bivalve species, particularly higher survival and growth rates (Vaughn and Spooner, 2006). Among bivalve species, native freshwater mussels are essential components of the structure and functioning of ecosystems (Vaughn and Hakenkamp, 2001). Freshwater mussels of the order Unionoida have been rapidly declining over the past century in several parts of the world, particularly in North America and Europe (Ricciardi *et al*., 1998; Vaughn and Taylor, 1999; Bogan, 2008; Simon *et al*., 2015; Lopes-Lima *et al*., 2017). The main causes pointed for the decline of this faunal group include the degradation and loss of habitat caused by human-made structures (*e.g.* dams, channels), poor agricultural practices, commercial exploitation for pearl culture, pollution, climate changes and introduction of NIS (Wilcove *et al*., 1998; Anthony and Downing, 2001; Dudgeon *et al*., 2006). The reproductive strategy of *C. fluminea,* characterized by high fecundity and rapid growth rate, may also constitute a competitive advantage because unionids have lower growth rates and a unique and complex lifecycle that requires a suitable fish host to incubate the glochidia, which constitutes the primary method of dispersal in these animals (Zanatta and Murphy, 2006). Although these impacts are well documented, it is still necessary to clarify the type and strength of the interactions between *C. fluminea* and native bivalve species in distinct ecosystems (Schmidlin and Baur, 2007). Several studies have been reporting the replacement of the native freshwater bivalves by invasive species such as *Dreissena polymorpha* in many ecosystems (Bódis *et al*., 2014a; Burlakova *et al*., 2014). Nevertheless, there is great speculation on the real impacts of *C. fluminea* over native mussels, and the contribution of *C. fluminea* for their decline is not well established (Vaughn and Spooner, 2006; Ferreira-Rodríguez *et al*., 2108). Some studies report the coexistence of dense populations of both native mussels and *C. fluminea* (Miller and Payne, 1994; Strayer, 1999).

However, other studies based on spatial distributions suggest that competition with *C. fluminea* may lead to the extirpation of native mussel populations (Kraemer, 1979; Elliott and Ermgassen, 2008). Thus, it is important to continue to investigate the potential link between the introduction of *C. fluminea* and the decline of native bivalve species (Ferreira-Rodríguez *et al*., 2018).

Despite the negative aspects described above, *C. fluminea* may also have positive impacts on the invaded ecosystems. Often abundant, the empty shells of *C. fluminea* are important structures to the organization of some macrobenthic communities (Bódis *et al*., 2014b; Crooks, 2002; Gutiérrez *et al*., 2003; Ilarri *et al*., 2015). They provide shelter to organisms escaping from predators or avoiding sources of environmental stress, fostering their abundance and diversity (Werner and Rothhaupt, 2007; Sampaio and Rodil, 2014; Novais *et al*., 2016). Due to its high filtration rates, *C. fluminea* consumes large amounts of phytoplankton, reducing eutrophication and increasing water clarity, which promotes the growth of submerged vegetation that can constitute an additional habitat for some invertebrate and fish species (Phelps, 1994). *C. fluminea* can serve as food for some predators, including some fish species and crayfish (Pereira *et al*., 2016).

C. fluminea bioinvasions can cause large economic losses (Pimentel *et al*., 2005). For example, its biofouling activity adversely affect human activities and infrastructures, causing serious damage on man-made structures of water-dependent industries such as electric power stations, water treatment plants, sand and cement industries and irrigation systems (Rosa *et al*., 2011). However, *C. fluminea* bioinvasions can also offer opportunities. The species is used for human consumption especially in Asia, and in China is one of the most economically important aquatic species (Chen *et al*., 2013). *C. fluminea* is highly appreciated mainly for its nutritional value and benefits as a healthy food with hepatoprotective, antihypertensive, hypocholesterolemic and antitumor properties (Chijimatsu *et al*., 2009, 2013; Liao *et al*., 2016; Peng *et al*., 2017).

Due to its feeding strategy, *C. fluminea* is able to uptake contaminants not only from the water column but also from the sediment. It can be used as a biofilter against chemical and biopatogens in aquaculture, ornamental fish production and maintenance, water clearance, and environmental bioremediation (Buttner, 1986; Graczyk *et al*., 2003; Miller *et al*., 2005; Rosa *et al*., 2014b; Erdoğan and Erdoğan, 2015; Silva *et al*., 2016). Therefore, their exploitation for several proposes in invaded ecosystems may be increased, helping to control the negative impacts of its bioinvasions.

Thus, investigating the dynamics of bioaccumulation and depuration of relevant environmental contaminants in this species is of major importance for the establishment of an environmental risk assessment, in regard of the effects in ecosystems and particularly in human health.

1.2.5. Use of *C. fluminea* in environmental studies

Environmental contaminants are continually released from anthropogenic sources to aquatic ecosystems, putting environmental and human health at risk (Johsnton *et al*., 2015). Heavy metals are of particular concern because they are widespread and persistent contaminants, and several of them are very toxic to aquatic organisms (Javed *et al*., 2017). Moreover, heavy metals can be accumulated by aquatic animals and some of them can also be biomagnified along the food chain, increasing the risk of exposure and toxic effects to humans consuming contaminated specimens (Jaishankar *et al*., 2014). Additionally, in polluted environments, heavy metals are often associated with other contaminants, constituting complex mixtures (Wu *et al*., 2016).

Well known effects of several metals include the ability to interfere in cellular processes causing imbalance in reactive oxygen species (ROS) production, enzyme inhibition and depletion of essential macromolecules of the antioxidant defence system (Lushchak, 2014). The overproduction of ROS can ultimately result in oxidative stress and damage of cellular components. The cell is equipped with enzymatic and non-enzymatic defence mechanisms operating to eliminate ROS and prevent oxidative damage. Alterations in the components of the antioxidant defence system have been used as sub-individual biomarkers in organisms under toxicant stress exposure (*e.g.* Vlahogianni and Valavanidis, 2007; Klimova *et al*., 2017). The enzymes catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx) constitute the first line of defence, eliminating several ROS including hydroxyl radicals, superoxide anion and hydrogen peroxide (Matés, 2000). Glutathione *S*-transferases (GST) belong to a family of biotransformation enzymes, catalysing the conjugation of electrophilic compounds with glutathione (GSH) for excretion (van der Oost *et al*., 2003). Despite the efficiency of this system, it is not always possible to prevent cellular damage and lipid peroxidation (LPO) is one of the possible outcomes. LPO involves a set of chain reactions that ultimately result in oxidation of polyunsaturated lipids (Vasilaki and McMillan, 2011). Lipid peroxides are responsible for membrane alterations which often precedes irreversible cellular damage. They are highly reactive compounds, which results in the generation of more ROS (Gaschler and Stockwell, 2017).

Several metals also have the ability to interfere with other biological systems related to vital functions such as the nervous system (Čolović *et al*., 2013). Cholinesterases (ChE) are a group of enzymes that belong to the esterase family. They include enzymes involved in the neurotransmission and in other functions, such as detoxification (Moser and Padilla, 2016). In vertebrates, they are divided in acetylcholinesterase and pseudocholinesterase, also known as non-true cholinesterase, butyrylcholinesterase and propyonylcholinesterase (Pezzementi *et al*., 2011). Acetylcholinesterase is the enzyme responsible for the hydrolysis of acetylcholine into choline and acetic acid, a crucial reaction for terminating impulse signalling at cholinergic synapses (Colletier *et al*., 2006). Pseudocholinesterases hydrolyse or bind to several endogenous toxic substances and xenobiotics, therefore being important in detoxification. The inhibition of ChE has been used as biomarker of neurotoxicity for a wide range of environmental contaminants, including metals (Guilhermino *et al*., 1998; 2000; Kopecka-Pilarczyk, 2010; Choi *et al*., 2011; de Lima *et al*., 2013). However, it is important to understand and characterize the type of ChE enzymes present in the species and tissues to be used in environmental studies as well as their physiological functions to avoid misinterpretations and bias (Garcia *et al*., 2000). This is even more important in studies with invertebrates because their ChE have differences in biochemical properties, physiological functions, and behaviour towards environmental contaminants in relation to the enzymes of vertebrates, and among of distinct tissues of the same species (Mora *et al*., 1999; Frasco *et al*., 2007; Ramos *et al*., 2012). The ChE present in the whole body of *C. fluminea* (Mora *et al*., 1999) and in different tissues of *C. fluminea* from the estuaries of M-est and L-est were characterized (Rocha, 2013). For example, in the adductor muscle that is responsible for the oppening and closing of the shell, evidences point to a single enzyme having properties of both typical acetylcholinesterase and pseudocholinesterase that is believed to be involved in cholinergic neurotransmission (Rocha, 2013).

The impact of metal exposure in the energetic metabolism can be assessed by measuring the alteration in activity of key enzymes of energetic metabolism (Ivanina *et al*., 2008). NADP-dependent isocitrate dehydrogenase (IDH) is an enzyme of the aerobic metabolism and also plays an important role as antioxidant against oxidative stress because provides NADPH to be used in the recycling of glutathione (Hegazi, 2010). Octopine dehydrogenase (ODH) is a key enzyme of the anaerobic metabolism which maintains redox balance under anaerobic conditions (Lima *et al*., 2007).

C. fluminea is an important bioindicator of heavy metal contamination because couples information of both pelagic and benthic compartments (Doherty, 1990; Patrick *et al*., 2017).

Additionally, it shows advantages for the use in ecotoxicological studies: it is widely distributed and abundant, easy to collect, can be maintained in laboratory conditions for several months, and the adults have sufficient amount of different tissues to perform multiple analyses. Because it is a NIS, there are no ethical constraints regarding its capture, thus it has been used as a surrogate for native species in environmental studies (Sherman *et al*., 2009; Lopes-Lima *et al*., 2014; Phelps, 2016; Bonnail *et al*., 2017). The first studies on the effects of metals on *C. fluminea* focused mainly on the accumulation and mortality (Doherty and Cherry, 1988; Doherty, 1990), but in the last decades a wide range of analytical and biochemical procedures were developed allowing the assessment of different effects of these substances at different levels of biological organization. Several aspects regarding metal toxicity in *C. fluminea* have been investigated including behavioural changes (valve closure and ventilatory activity), histological alterations and effects in gene expression and in enzymatic activities, among others (Table 1). Metallothioneins are low-molecular-weight cytosolic proteins that participate in the homeostatic control of essential metals and in the detoxification of non-essential metals (Marie *et al*., 2006a) and whose induction was observed in *C. fluminea* exposed to arsenic, copper, cadmium and zinc (Table 1). The induction of a multixenobiotic resistance mechanism was also observed as response to arsenic, copper, lead, mercury, uranium and zinc (Table 1). This mechanism is mediated by membrane proteins that pump chemicals out of the cell (Kurelec, 1992).

Table 1. Parameters evaluated in *Corbicula fluminea* exposed to heavy metals in field (F) and laboratory (L) studies. Catalase (CAT); Cyclooxygenase 1 (cox1); Glutathione, reduced form (GSH); glutathione peroxidase (GPx); Glutathione *S*-transferases (GST); Heat shock protein (Hsp); Lipid peroxidation (LPO); Messenger ribonucleic acid (mRNA); Metallothioneins (MT); Multixenibiotic resistance (MXR); Peroxidase (POD); Retinoblastoma gene (RB); selenium-dependent glutathione peroxidase (Se-GPx); 12S ribosomal RNA (12S); Ribosomal S9 protein gene (*rps9*); Sodium potassium adenosine triphosphatase (Na^+/K^+-ATPase); Superoxide dismutase (SOD).

Substance	Study	Parameter	Reference
Arsenic	F	Accumulation	Abaychi *et al.*, 1988
	F	MXR protein	Achard *et al*, 2004
	L	Expression of *rpS9* gene	Achard-Joris *et al.*, 2006
	F	Accumulation	Andres *et al.*, 1999
	F	Accumulation	Arini *et al.*, 2011
	L	Growth, mortality, MT, ultrastructural alterations of gills and mantle	Baudrimont *et al.*, 1997
	F	MT	Baudrimont *et al.*, 1999
	L	Lysosomal system, mRNA levels of SOD, CAT, GPx and GST, MT transcript levels	Bigot *et al.*, 2011
	L	Phagocytic activity; haemocytes, lysosomal alterations	Champeau *et al.*, 2007
	L	Accumulation, valve closure	Chen *et al.*, 2010
	L	Uptake, mortality	Chen and Liao, 2012
	L	Uptake, mortality	Costa *et al*., 2009
	L	MT	Diniz *et al.*, 2007
	L	MT	Doherty *et al.*, 1998
	L	Low molecular weight antioxidants, lipid soluble antioxidants, Tissue radical absorption capacity, histological alterations	Legeay *et al.*, 2005
	L	Mortality	Liao *et al.*, 2008
	L	MT, histological and histochemical alterations	Santos *et al.*, 2007
	F	Accumulation	Sebesvari *et al.*, 2005

Table 1. (Continued)

Substance	Study	Parameter	Reference
Cadmium	F+L	Accumulation, condition index, MT, gene expression of cat, sodMn, gst, 12S, cox1 and mt.	Arini *et al*., 2014
	L	Uptake	Fraysse *et al.*, 2000
	L	Effect on uptake and depuration of 57Co, 110mAg and 134 Cs	Fraysse *et al.*, 2002
	L	Phosphoadenylate, adenylate energy charge	Giesy *et al.*, 1983
	L	Accumulation	Graney *et al.*, 1984
	L	Accumulation	Inza *et al.*, 1997
	L	Uptake and elimination	Inza *et al.*, 1998
	F	Valve closure	Liao *et al.*, 2005
	F	Accumulation	Lu *et al.*, 2011
	F	MT	Marie *et al.*, 2006a
	F	MT	Marie *et al.*, 2006b
	L	Uptake and elimination	Qiu *et al.*, 2005
	L	SOD, POD, GSH	Ren *et al.*, 2013
	F	Accumulation	Ruelas-Inzunza *et al.*, 2009a
	F	Trophic transfer	Simon *et al.*, 2000
	L	Condition index, Composition of low molecular weight metabolites	Spann *et al.*, 2011
	F	Accumulation	Tran *et al*., 2001
	F	Ventilatory activity	Tran *et al*., 2002
	L	Valve closure	Tran *et al*., 2003a
	F	Accumulation	Villar *et al*., 1999

Table 1. (Continued)

Substance	Study	Parameter	Reference
Chromium	F	Accumulation	de Oliveira and Martinez (2014)
	L	Accumulation, oxidative response	Wang *et al*., 2012
Copper	F	MXR	Achard *et al*., 2004
	L	Tissue and shell growth	Belanger *et al.*, 1990
	L	Lysosomal system, mRNA levels of SOD, CAT, GPx and GST and MT transcripts	Bigot *et al.*, 2011
	L	Accumulation and elimination	Croteau *et al.*, 2004
Lead	L	Accumulation pathways	Croteau and Luoma, 2005
	L	Valve closure	Jou *et al.*, 2009
	F	Valve closure	Liao *et al.*, 2005
	L	Accumulation	Netpae and Phalaracksh, 2009
	F	Accumulation	Ruelas-Inzunza *et al.*, 2009a
	L	Valve closure	Tran *et al.*, 2003b
	F	Accumulation	Villar *et al.*, 1999
	F	MXR protein	Achard *et al.*, 2004
	L	Lysosomal system, mRNA levels of SOD, CAT,GPx and GST and MT transcripts	Bigot *et al.*, 2011
	L	Accumulation and elimination	Croteau *et al.*, 2004
	L	Accumulation pathways	Croteau and Luoma, 2005
	F	Accumulation	de la Cruz *et al*., 2017
	L	Valve closure	Jou *et al.*, 2009
	F	Valve closure	Liao *et al.*, 2005
	L	Accumulation	Netpae and Phalaracksh, 2009
	F	Accumulation	Ruelas-Inzunza *et al.*, 2009a
	L	Na^+/K^+ ATPase, carbonic anhydrase activities, MXR protein, accumulation of RB, expression of P-glycoprotein and Hsp70	Rocha and Souza, 2012

Table 1. (Continued)

Substance	Study	Parameter	Reference
Mercury	F	MXR	Achard *et al.*, 2004
	L	Growth, mortality, MT and ultrastructural alterations of gills and mantle	Baudrimont *et al.*, 1997
	F	SOD, CAT, GR, GPx-Se, GST, GSH, MT, DNA strand breaks and LPO	Faria *et al.*, 2010
	F	Accumulation	Gentès *et al.*, 2013
	L	Accumulation, tissue distribution	Inza *et al.*, 1997
	L	Uptake and elimination	Inza *et al.*, 1998
	F	Accumulation	Paller *et al.*, 2004
	F	Accumulation	Ruelas-Inzunza *et al.*, 2009b
	L	Trophic transfer	Simon *et al.*, 2000
	F	Accumulation	Schmitt *et al*., 2011
	L	DNA strand breaks	Westerfield *et al.*, 1996
Uranium	L	Accumulation, mortality	Labrot *et al.*, 1999
	L	Accumulation	Simon *et al.*, 2004
	L	Genotoxic damage	Simon *et al.*, 2011
	L	Accumulation	Tran *et al.*, 2004
	L	MXR protein; Hsp60	Tran *et al.*, 2005
	L	Accumulation	Tran *et al.*, 2008
Zinc	F	MXR	Achard *et al.*, 2004
	L	Gene expression of ribosomal S9 protein gene	Achard-Joris *et al.*, 2006
	F+L	Accumulation, condition index, MT and gene expression of cat, sodMn, gst, 12S, cox1 and mt.	Arini *et al*., 2014
	F	Accumulation	Andres *et al.*, 1999
	F	Accumulation	Arini *et al.*, 2011

Table 1. (Continued)

Substance	Study	Parameter	Reference
Zinc	F	Accumulation	Angelo *et al.*, 2007
	F	MT	Baudrimont *et al.*, 1999
	F+L	MT	Baudrimont *et al.*, 2003
	F+L	Growth	Belanger *et al*., 1986
	L	Growth; cellulolytic activity	Farris *et al.*, 1989
	L	Effect on uptake and depuration of 57Co, 110mAg and134Cs	Fraysse *et al.*, 2002
	F	MT	Marie *et al.*, 2006b
	L	Uptake and elimination	Qiu *et al.*, 2005
	F	Accumulation	Ruelas-Inzunza *et al.*, 2009a
	L	Condition index and low molecular weight metabolites	Spann *et al.*, 2011
	F	Accumulation	Villar *et al.*, 1999

Among heavy metals, mercury is one that causes major concern due to its high toxicity to animals and humans (UNEP, 2013). It is a priority hazardous substance (EC, 2008a) under the Water Framework Directive (EC, 2000) and is also listed as a priority pollutant by the United States Environmental Protection Agency and by the Oslo and Paris Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR Convention). Recognizing the hazards posed by mercury to human environment health, the Minamata Convention was created to implement measures to reduce and regulate anthropogenic emissions of mercury (UNEP, 2013).

Mercury is a naturally occurring element on Earth that can reach aquatic ecosystems through different ways that include natural processes of erosion and volcanic activity. Nevertheless, human activities including industry and mining have been increasing the mercury levels in several areas (Tchounwou *et al*., 2012).

In the environment, mercury exists in multiple oxidative states, as elemental mercury, inorganic salts and organic complexes, each one presenting different toxicity profiles (Guzzi and La Porta, 2008). Inorganic forms are predominant in environmental compartments (water, soil and sediment) while organic ones are dominant in the biota (Beckers and Rinklebe, 2017). Both forms have high toxicity to aquatic life and humans and are accumulated by a high range of organisms (Zahir *et al*., 2005; Frasco *et al*., 2007, 2008; Vieira *et al*., 2009; Cardoso *et al*., 2013; Harayashiki *et al*., 2018; Nowosad *et al*., 2018). Moreover, organic forms, especially methylmercury, are biomagnified in trophic chains (Lavoie *et al*., 2013). At cellular level, mercury toxicity is related to the high affinity for sulphydril and thiol groups present in macromolecules such as cysteine and glutathione (Ballatori and Clarkson, 1985) and in several enzymes (Waku and Nakazawa, 1979). Mercury enhances the production of ROS inducing oxidative stress and cellular damage including LPO (Lund *et al*., 1991), DNA damage (Pereira *et al*., 2010), and alterations in membrane permeability (Ballatori *et al*., 1988).

Mercury is known to be highly accumulated by *C. fluminea* (Paller *et al*., 2004; Gentès *et al*., 2013) (Table 1), and the mechanisms of uptake and elimination as well as the effect of temperature and pH in these processes were previously investigated (Inza *et al*., 1998). The involvement of defence mechanisms in response to mercury exposure was observed in *C. fluminea* and includes the induction of a multixenobiotic resistance protein (Achard *et al*., 2004) and enzymes of the antioxidant system, namely CAT and superoxide dismutase SOD (Faria *et al*., 2010) (Table 1). However, further studies on the effects of mercury in this species are needed, including in relation to energy production pathways, neurotransmission and parameters indicative of individual fitness such as oxygen consumption rate and filtration rate.

Among emerging environmental contaminants, microplastics are of special concern (EPA, 2017). Microplastics are plastic particles with size less than 5 μm, with various shapes and colours, resulting from the breakdown of larger plastic fragments in the environment or introduced into ecosystems already in the micro or nano scale due to their used in several products such as cosmetics and personal care products, electronic equipment and synthetic textile fibers, among several others (Napper *et al*., 2015; De Falco *et al*., 2018). They are considered a serious global problem that has been raising public and scientific awareness in relation to environmental and human health (Eriksen *et al*., 2014). For this reason they were included in the list of descriptors of the Marine Strategy Framework Directive (Descriptor 10 - Marine Litter) (EC, 2008a). Although microplastics are very abundant in freshwater ecosystems (Li *et al*., 2018) the knowledge of their effects on freshwater organisms is still limited. Nevertheless, studies report several effects on freshwater organisms, including physical impacts, transference of adsorbed chemicals, tissue and cellular damage, alterations in metabolic function and immune system (Eerkes-Medrano *et al*., 2015; Horton *et al*., 2017; Li *et al*., 2018). The effects of microplastics in *C. fluminea* were previously investigated (Rochman *et al*., 2017; Guilhermino *et al*., 2018;) providing a basis for further studies on the combined effects of this contaminant with other substances in this species. Since microplastics can occur simultaneously with other environmental contaminants including mercury, investigating possible toxicological interactions between these substances is of major interest.

1.3. Objectives and Outline of the Thesis

The central aim of the present Thesis was to investigate the effects of environmental contaminants on the exotic invasive bivalve, *C. fluminea*. The species has a high invasive capability with adverse environmental impacts, has a wide geographical distribution and is consumed as food by humans. Thus, studying the effects of priority global pollutants on *C. fluminea* is of the utmost importance regarding environmental and human health.

In view of the above considerations, four specific questions (SQ) were formulated:

SQ1: Does the acclimation time period influence the baseline levels of selected biomarkers in *C. fluminea*?

SQ2: Is *C. fluminea* less sensitive to acute (96 h) exposure to mercury than one of its native bivalve competitors (*Anodonta anatina*)

SQ3: Do the environmental conditions of the natural habitat influence the sensitivity of *C. fluminea* to mercury and its post-exposure recovery?

SQ4: Does the presence of microplastics influence the toxicity, post-exposure recovery and bioconcentration of mercury in *C. fluminea*?

The present Thesis is organized in six Chapters: the Chapter I corresponds to the general introduction; the Chapters II to V correspond to the experiments performed to answer the specific questions and attain the main objective of the Thesis; the Chapter VI corresponds to the general discussion and conclusions, and the Chapter VI is the list of references.

In the general introduction (Chapter I), the problem of bioinvasions is introduced and the main aspects of the biology, ecology, ecotoxicology and impacts of *C. fluminea* are reviewed.

The main goal of Chapter II, entitled "Acclimation conditions for the use of the exotic invasive species *C. fluminea* in toxicity bioassays" was to answer the SQ1: "Does the acclimation time period influence the baseline levels of selected biomarkers in *C. fluminea*?" The rationale for SQ1 is that the adaptation to laboratory conditions may influence the levels of *C. fluminea* biomarkers potentially leading to bias in the interpretation of the information provided when such parameters are used as effect criteria in toxicity bioassays using specimens from wild populations. The selected biomarkers (ChE, IDH, ODH, CAT, GR, GPx, GST activities and LPO levels) were determined at the arrival of organisms to laboratory, and after 7 and 14 days of acclimation. The results show that after 7 days all biomarkers except ODH were significantly altered, indicating stress under the new conditions. After 14 days, they returned to levels determined at the arrival to the laboratory. Thus, a period of 14 days seems to be an adequate acclimation period before toxicity bioassays and was selected for use in the further experiments.

The main objective of Chapter III was to answer the SQ2: "Is *C. fluminea* less sensitive to acute (96 h) exposure to mercury than one of its native bivalve competitors (*Anodonta anatina*)?" For that, the sensitivities of *C. fluminea* and *A. anatina* (one of its native competitors in European ecosystems) to mercury exposure were compared. In laboratory bioassays, specimens of *C. fluminea* and *A. anatina* from the M-est and from the Tâmega River, respectively, were independently exposed for 96 h to clean medium (control) and five mercury concentrations (31, 63, 125, 250 and 500 µg/L). The effect criteria were: mortality, adductor muscle ChE activity (indicative of neurotoxicity), foot IDH and ODH activities (related to aerobic and anaerobic pathways of energy production), the activities of gill CAT, GR, GPx and GST (enzymes of the antioxidant system) and LPO

levels (indicative of oxidative damage). Mercury caused high mortality in *A. anantina* (72 h LC_{50} = 49.6 µg/L), whereas no mortality was observed in *C. fluminea* up 500 µg/L. Thus, *C. fluminea* was less sensitive to mercury than *A. anatina*. During the exposure period, *C. fluminea* closed the shells for long periods of time avoiding toxicant exposure and decreased the aerobic production of energy, as suggested by the significant reduction of IDH activity. Moreover, some antioxidant defences were activated preventing the occurrence of lipid peroxidation damage up to 63 µg/L. These results indicate that mercury may modulate the competition between *C. fluminea* and *A. anatina* in ecosystems contaminated with the metal, acting in favour of the NIS, which is more tolerant than the native species. Therefore, it is important to further investigate the mechanisms involved in the relative tolerance of *C. fluminea* to mercury, especially under longer exposure at ecologically relevant concentrations, as well as its recovery capacity after mercury exposure events.

The work presented in Chapter IV was performed to answer the SQ3 “Do the environmental conditions of the natural habitat influence the sensitivity of *C. fluminea* to mercury and its post-exposure recovery?” To answer this question, the effects induced by 8 days and 14 days of exposure to mercury and the post-exposure recovery in *C. fluminea* from two estuaries with several environmental differences were compared. Bivalves collected in the M-est and in the L-est were acclimated to laboratory conditions for 14 days. Then, groups of bivalves from the two estuaries were independently exposed to clean medium for 8 or 14 days (controls), 31 µg/L of mercury for 8 or 14 days, and to 31 µg/L of mercury for 8 days + 6 days to clean medium (post-exposure recovery). The effect criteria were the biomarkers used in the previous studies and the oxygen consumption rate (OCR). After 8 days, mercury caused effects in bivalves from the M-est but not in those of the L-est. Moreover, evidences of delayed toxicity induced by 8 days of exposure to mercury were found but only in bivalves from the M-est. After 14 days of exposure to mercury, animals from both estuaries had significantly reduced OCR and inhibited IDH activity. Bivalves from M-est showed significant oxidative stress and lipid peroxidation damage, whereas these effects were not found in L-est bivalves. Moreover, bivalves from L-est showed a higher recovery capacity in OCR and IDH activity. Thus, M-est bivalves were more sensitive to mercury than those of the L-est. Because the acclimation and the exposure conditions were the same, these findings indicate that the environmental conditions of the natural habitats to which the bivalves were exposed in pre-developmental phases influence their sensitivity to mercury.

In Chapter V was investigated the effects of a mixture of microplastics and mercury in *C. fluminea*, the post-exposure recovery and the potential influence of microplastics in mercury bioconcentration by this species. The objective was to answer SQ4 “Does the

presence of mercury influence the toxicity, post-exposure recovery and bioaccumulation of mercury in *C. fluminea*?" The rationale is that both microplastics and mercury are global pollutants occurring simultaneously in several ecosystems. Therefore, in such ecosystems the biota is simultaneously exposed to the two environmental contaminants and toxicological interactions may occur. To answer the question, *C. fluminea* specimens from the M-est were exposed in laboratory conditions to the following treatments: 8-day exposure to clean medium (control) ; 8-day exposure to 0.13 mg/L of microplastics; 8-day exposure to 0.03 mg/L of mercury; 8-day exposure to a mixture of microplastics (0.13 mg/L) and mercury (0.03 mg/L), hereafter indicated as mixture; 14-d control; 8-day exposure to 0.13 mg/L of microplastics + 6-day exposure to clean medium; 8-day exposure to 0.03 mg/L of mercury + 6-day exposure to clean medium; and 8-day exposure to the mixture + 6-day in clean medium. The bioconcentration factor of mercury in bivalves exposed for 8 days to mercury alone was significantly higher than that determined in animals exposed to the mixture. Mercury alone caused a significant decrease in the filtration rate, in IDH, GR and GPx activities, as well as a significant increase in CAT and GST activities and in LPO levels. After 8 days of exposure to microplastics alone, microplastic particles were found in the digestive tract and in the gills. These animals had significant decreased filtration rate, inhibited ChE, and increased LPO levels. The mixture caused a significant decrease in the filtration rate, inhibition of GR and GPx activities and increased CAT activity and LPO levels. The recovery period after microplastics, mercury and mixture exposures, was not effective for all biomarkers. Overall, the results of this study indicate that microplastics influence the bioaccumulation and toxicity of mercury to *C. fluminea* and suggest antagonism between the two pollutants in this species.

Chapter VI is the general discussion of the main findings obtained in the experiments and the conclusions that can be taken.

CHAPTER II

Acclimation conditions for the use of the exotic invasive species *Corbicula fluminea* in toxicity bioassays

Abstract

Capture, transport, handling and maintenance of wild animals in laboratory conditions may be factors of stress potentially influencing the results of toxicity tests. To avoid such problems or to minimize their influence, animals captured in the wild are in general acclimated to laboratory conditions before being used in bioassays. Thus, with the ultimately goal of using *Corbicula fluminea* from wild populations in toxicity bioassays, the objective of this study was to determine the time required to the levels of biomarkers commonly used as effect criteria in toxicity bioassays return to baseline values in this species under specific laboratorial conditions. Animals collected in the upper part of Minho River estuary were transported to the laboratory. In 9 animals, samples of the adductor muscle, foot and gills were collected (day 0) for determination of a battery of sub-individual biomarkers. The remaining animals were acclimated to a temperature of 16 ± 1 ºC, photoperiod 16 h light: 8 h dark and they were fed every 48 hours with a mixture of microalgae. After 7 and 14 days in these conditions samples for sub-individual biomarkers determination were collected in groups of 9 animals. Relatively to day 0, after 7 days of acclimation, animals had significantly ($p \leq 0.05$) increased activity of the enzymes cholinesterases, NADP-dependent isocitrate dehydrogenase and catalase, significantly increased ($p \leq 0.05$) lipid peroxidation levels, and significantly ($p \leq 0.05$) decreased activity of the enzymes glutathione reductase, glutathione peroxidase and glutathione *S*-transferases. After 14 days, no significant ($p > 0.05$) differences in relation to day 0 were found in any of the studied biomarkers. Therefore, is recommended a 14-day period of acclimation to laboratory conditions *of C. fluminea* from wild populations before its use in toxicity bioassays using these biomarkers as effect criteria.

Key Words: *Corbicula fluminea*, Biomarkers, Laboratory acclimation, Toxicity tests

2.1. Introduction

Toxicity tests are fundamental tools to assess the impacts of chemical substances and other stressors on living organisms (Krewski *et al*., 2010). Before use in controlled experiments, animals from wild populations are often subjected to an acclimation period in controlled laboratory conditions, since their collection, transport and transference to new environmental conditions can be stressful events and induce changes in several physiological parameters (Má *et al*., 2011). In situations of stress, organisms display a series of adaptive responses towards homeostasis (Obernier and Baldwin, 2006). One of the key aspects of toxicity bioassays is to ensure that alterations in endpoints are due to the stressor(s) under study, thus acclimation to laboratory conditions is essential to reduce stress and deviation from the homeostasis, and to stabilize physiological parameters to baseline levels (Thompson *et al*., 2012). This is essential to attribute the effects observed to the chemical(s) or other factors being tested and not to variables that are not being assessed (*e.g.* temperature, salinity and water dissolved oxygen). Such variations create confounding results, often difficult data interpretation and ultimately may lead to wrong conclusions (Vidal *et al*., 2002a, 2002b; Troschinski *et al*., 2014).

Biomarkers have been used as practical tools for assessing early biological effects of chemical contaminants (Hagger *et al*., 2006). Nevertheless, although presenting several advantages, they can be affected by environmental variables (Lee *et al*., 2015). In view of this limitation, a careful planning of toxicological tests is necessary to minimize the interference of external variables that may lead to incorrect interpretations of results.

Corbicula fluminea is one of the most successful non-indigenous species (NIS) of aquatic ecosystems, causing important ecological and socio-economic impacts (Coelho *et al*., 2018; Laverty *et al*., 2015). In some areas of its native range it is an important commercial species, used for human consumption (Chen *et al*., 2013).

For this reason *C. fluminea* has been widely investigated regarding its ecology, abundance in distribution, ecological impacts and effects of environmental contaminants, among other aspects (Sousa *et al*., 2008a; Patrick *et al*., 2017; Guo and Feng, 2018). Thus, it is important to establish an ideal acclimation period for *C. fluminea* to be used in toxicity bioassays. The biomarkers selected for this study are related to crucial functions including nerve impulse transmission, energy production, antioxidant defences, and oxidative damage. The activities of cholinesterase enzymes (ChE), NADP-dependent isocitrate dehydrogenase (IDH), octopine dehydrogenase (ODH), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione *S*-transferases (GST), and lipid peroxidation (LPO) levels have been previously employed in field and

laboratory studies with *C. fluminea* (Bonnafé *et al*., 2015; Cid *et al*., 2015; de Oliveira *et al*., 2016; Guilhermino *et al*., 2018).

2.2. Material and methods

2.2.1. Chemicals

The chemicals used in biomarkers determinations were of analytical grade and purchased from Sigma-Aldrich Chemical (Germany), Merck (Germany) and Bio-Rad (Germany).

2.2.2. Collection of animals and transport to the laboratory

C. fluminea adults were collected in the upper part of Minho River estuary (NW of the Iberian Peninsula), hereafter indicated as M-est, a relatively low impacted estuary that is included in NATURA 2000. Animals were collected in one site (~42°30'22.51''N, 8°32'22.51''W) at low tide with an adapted rake. After collection they were transported to the laboratory within the lowest time possible in aerated thermal boxes partially filled with water from the sampling site.

2.2.3. Experimental conditions and sample collection

In the laboratory, bivalves were measured with a calliper and a sample of 48 specimens having a mean anterior-posterior shell length of 29.6 ± 1.1 mm was selected for the experiment. From these, 9 animals were immediately sacrificed (T_0), and the following tissues were isolated on ice: the adductor muscles were placed in 1 mL of potassium phosphate buffer (0.1 M, pH = 7.2). One foot portion was put in 1 mL of tris(hydroxymethyl)aminomethane buffer (Tris buffer) (0.5 M; pH = 7.8) and the other piece was put in 1 mL of Tris buffer (0.5 M, pH = 7.5) with ethylenediaminetetraacetic acid disodium salt dihydrate (Na_2-EDTA) 0.1M and DL-1, 4-Dithiothreitol (DTT) 0.1M. The gills were placed in potassium phosphate buffer (0.1 M, pH = 7.4) in a 1:10 ratio (weight/volume). The samples were stored at −80 °C until the day of biomarkers analyses. The remaining bivalves were acclimated in a room with controlled temperature (16 ± 1°C) and photoperiod (16 h light: 8 h dark). The acclimation tanks consisted in 20 L transparent propylene boxes (39 cm × 28 cm × 28 cm) containing 16 L of dechlorinated tap water for human consumption (hereafter indicated as clean medium) constantly aerated by air bubble diffusers. Sixteen animals were randomly assigned to each acclimation tank. Every 48 hours the clean medium was renewed and the animals were fed with a 50%: 50% cells/cells mixture of *Chlorella vulgaris* and *Chlamydomonas reinhardtii* obtained from laboratorial cultures, with a final concentration of 8×10^5 algae cells/ml/bivalve. This

combination of microalgae was shown to be suitable for *C. fluminea* (Foe and Knight, 1986). After 7 days (T_7), 9 animals were randomly selected and sacrificed. Samples of the tissues previously indicated were isolated on ice and stored as previously described. The procedure was repeated after 14 days of acclimation (T_{14}). Clean medium temperature, pH, conductivity and dissolved oxygen were checked daily before and after medium renewals with a multi-parametric probe (HACH, Multi HQ 40d).

2.2.4. Analyses of biomarkers

On the day of biomarkers analyses, the gills were unfrozen on ice in the correspondent buffer and homogenized on ice (Ystral GmbH d-7801 homogenizer, Germany), in pulses of 10 seconds for 1 minute to minimize samples heating. One part of the gill homogenate (250 µL) was used to assess LPO levels according to Ohkawa *et al*. (1979) and Bird and Draper (1984) with adaptations (Filho *et al*., 2001; Torres *et al*., 2002). The quantification of thiobarbituric acid-reactive substances (TBARS), formed as a by-products of lipid peroxidation, was made spectrophotometrically at 535 nm in a cuvette with 200 µL of gill homogenate with Tris-HCl buffer (60 mM, pH 7.4) with diethylenetriaminepentacetic acid 0.1 mM, trichloroacetic acid 12% and 2-thiobarbituric acid 0.73%. The protein content of samples was determined in the remaining 50 µL of homogenate. The protein concentration of samples was determined at 600 nm, according to the Bradford method (Bradford, 1976) adapted to microplate by Frasco and Guilhermino (2002), using bovine *γ*-globulin as standard and a Bio-Rad Protein Assay solution prepared in ultra-pure water.
The remaining gill homogenate was centrifuged at 10000 ×*g* for 20 minutes at 4 ºC (Sigma Laboratory Centrifuge 3K30, Germany). Subsequently, the supernatant was carefully collected and its protein concentration was determined and standardized to 4 mg/mL. This fraction was distributed to different microtubes for determination of the activities of the antioxidant enzymes CAT, GR, GPx and GST. CAT activity was determined in a cuvette with 50 µL of supernatant, 950 µL of potassium phosphate buffer (0.05 M, pH = 7.0) and 500 µL of hydrogen peroxide (H_2O_2) 0.03 M. The decomposition of H_2O_2 in molecular oxygen and water was followed for 1 minute, at 240 nm, as proposed by Clairborne (1985). GR activity was assessed according to Carlberg and Mannervik (1985). To 100 µL of supernatant were added 900 µL of a reaction buffer consisting of nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), L-glutathione, oxidized form (GSSG), and diethylenetriaminepentaactic. The consumption of NADPH was monitored for 1 minute at 340 nm. GPx activity was determined according to Mohandas *et al*. (1984). The reaction consisted in 90 µL of supernatant, 800 µL of potassium phosphate buffer (0.05 M, pH = 7.0) prepared with Na_2-EDTA 1 mM, sodium azide 1 mM and GR 1 unit/mL, 50 µL of glutathione, reduced form (GSH) 4 mM, 50 µL

NADPH and 10 µL of H_2O_2. The NADPH decrease was followed 340 nm for 1 minute. The assessment of GST activity was made according to Habig *et al*. (1974) adapted to microplate by Frasco and Guilhermino (2002). To 50 µL of supernatant were added 250 µL of a reaction buffer consisting in potassium phosphate buffer (0.1 M, pH = 6.5), 1-chloro-2,4-dinitrobenzene 60 mM and GSH solution. The production of dinitrophenyl thioether was monitored at 340 nm for 1 minute.

The adductor muscles were homogenized in 1 mL of cold potassium phosphate buffer (0.1 M; pH = 7.2) on ice, and the homogenate was centrifuged at 3300 ×*g* for 3 minutes at 4º C. The supernatant was carefully collected, its protein concentration was determined as previously indicated, and standardized to 1 mg/mL. ChE activity was determined following the method of Ellman *et al.* (1961) adapted to microplate (Guilhermino *et al*., 1996). To 50 µL of adductor muscle supernatant were added 250 µL of a reaction buffer consisting of acetylthiocholine solution 0.075 M and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) 10 mM. The rate of production of 5-thio-2-nitrobenzoic acid was measured at 412 nm for 5 minutes.

Foot samples for IDH and ODH determinations were homogenized in Tris buffer (50 mM, pH = 7.8) and in Tris buffer (20 mM, pH = 7.5), respectively, with Na_2-EDTA 1 mM and DTT 1 mM, respectively. The homogenates were then centrifuged at 3300 ×*g* for 3 minutes at 4 ºC. The supernatants were collected and their protein content was determined (as previously indicated) and standardized to 1 mg/mL. IDH activity was assessed according to Ellis and Goldberg (1971) adapted to microplate by Lima *et al*. (2007) in 50 µL of sample with 200 µL of reaction buffer (Tris buffer (50 mM, pH = 7.8) with manganese(ii)-chloride-tetrahydrate 2 mM and DL-isocitric acid 7 mM and 50 µL of nicotinamide adenine dinucleotide, oxidized form ($NADP^+$) 0.5 mM solution. The production of NADPH was monitored for 3 minutes, at 340 nm. ODH activity was determined as suggested by Livingstone *et al*. (1990) adapted to microplate by Lima *et al*., (2007) in 50 µL of supernatant with 200 µL of reaction buffer (Tris buffer (20 mM, pH = 7.5), with EDTA 1 mM, DTT 1 mM, L-Arginine 5 mM and NADH 0.24 mM) and 50 µL of sodium pyruvate 5 mM solution. The consumption of pyruvate due to NADH oxidation was followed at 340 nm for 3 minutes.

For biomarker analyses and protein determinations at least three measurements were made for each sample and the respective blanks.

After biomarker determinations, the protein concentration of the samples was determined again and used to express the enzymatic activities and the LPO levels. LPO levels were expressed as nanomoles of TBARS per mg protein (nmol TBARS/mg protein). In all enzymatic determinations, the slope of the linear part of the reaction curve was used. Enzymatic activities were expressed in nanomoles per minute per mg of protein

(nmol/min/mg protein), except CAT that was expressed in micromoles per minute per mg of protein (µmol/min/mg protein). All the analyses were performed at 25 ºC in a Spectramax® M2 spectrophotometer (Molecular Devices, U.S.A.).

2.2.5. Statistical analysis

Each biomarker data set was tested for normality and homogeneity using the Shapiro-Wilk and Levene's tests, respectively, and analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's multi-comparison test when significant differences were found (Zar, 2010). GST and CAT data sets were transformed (square root transformation) to fulfil ANOVA assumptions. The significant level was 0.05. Analyses were performed using the SPSS statistics version 22.0 for Windows (IBM®, U.S.A.).

2.3. Results and discussion

No mortality was recorded during the experiment. Significant differences in the activity of the enzymes ChE, IDH, CAT, GR, GPx and GST and in LPO levels among different exposure times were found (Table 2).

Table 2. Results of the one-way ANOVA carried out with each biomarker data set of *Corbicula fluminea* to investigate the effect of the acclimation period. Cholinesterase enzymes (ChE) activity; NADP-dependent isocitrate dehydrogenase (IDH) activity; Octopine dehydrogenase (ODH) activity; Catalase (CAT) activity; Glutathione reductase (GR) activity; Glutathione peroxidase (GPx) activity; Glutathione *S*-transferases (GST) activity; Lipid peroxidation (LPO) levels; df - Degrees of freedom.

Biomarker	df	F	p
ChE	(2, 27)	5.384	0.008
IDH	(2, 27)	7.757	0.005
ODH	(2, 27)	1.902	0.172
CAT	(2, 27)	5.498	0.036
GR	(2 ,27)	40.87	0.000
GPx	(2, 27)	4.576	0.044
GST	(2, 27)	8.345	0.001
LPO	(2 ,27)	3.908	0.028

With the exception of ODH, all biomarkers levels determined at T_7 were significantly different from those determined at T_0 (Figs. 2 and 3). At T_7 the activities of ChE, IDH and CAT, and the LPO levels were significantly increased (37%, 61%, 46% and 48%, respectively) in relation to those determined at T_0. Not excluding the possibility of

interference from other variables (*e.g.* food type and abundance, water chemistry) the induction of adductor muscle ChE activity (Fig. 2A) could be related to the adaptation of organisms to increased temperature, as the water temperature at the collection site was 9.5 °C and in the temperature of clean medium in the acclimation tanks was 16 ± 1 °C. In a study investigating the seasonal variations of biomarkers in *C. fluminea*, the higher activities of soft entire body ChE were found in the warmer months (Vidal *et al.,* 2002b).

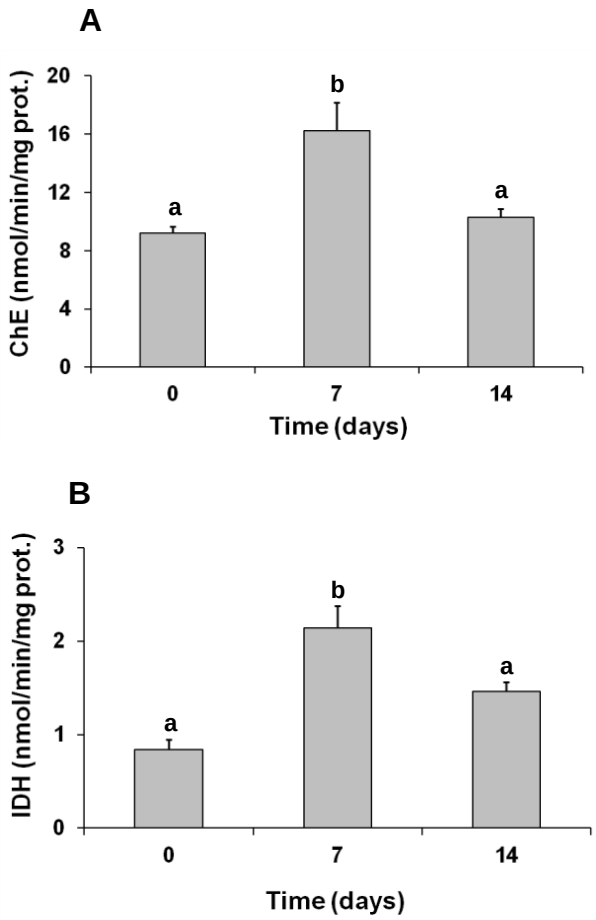

Fig. 2. A - Activity of cholinesterase enzymes (ChE) and B - Activity of NADP-dependent isocitrate dehydrogenase (IDH) of *Corbicula fluminea* determined after arrival to laboratory (day 0) and after acclimation to laboratory conditions for 7 and 14 days. The values are the mean ± standard error of the mean of 9 organisms. Significant differences between treatments are identified by different letters above the bars (one-way ANOVA and the Tukey's test, $p \leq 0.05$).

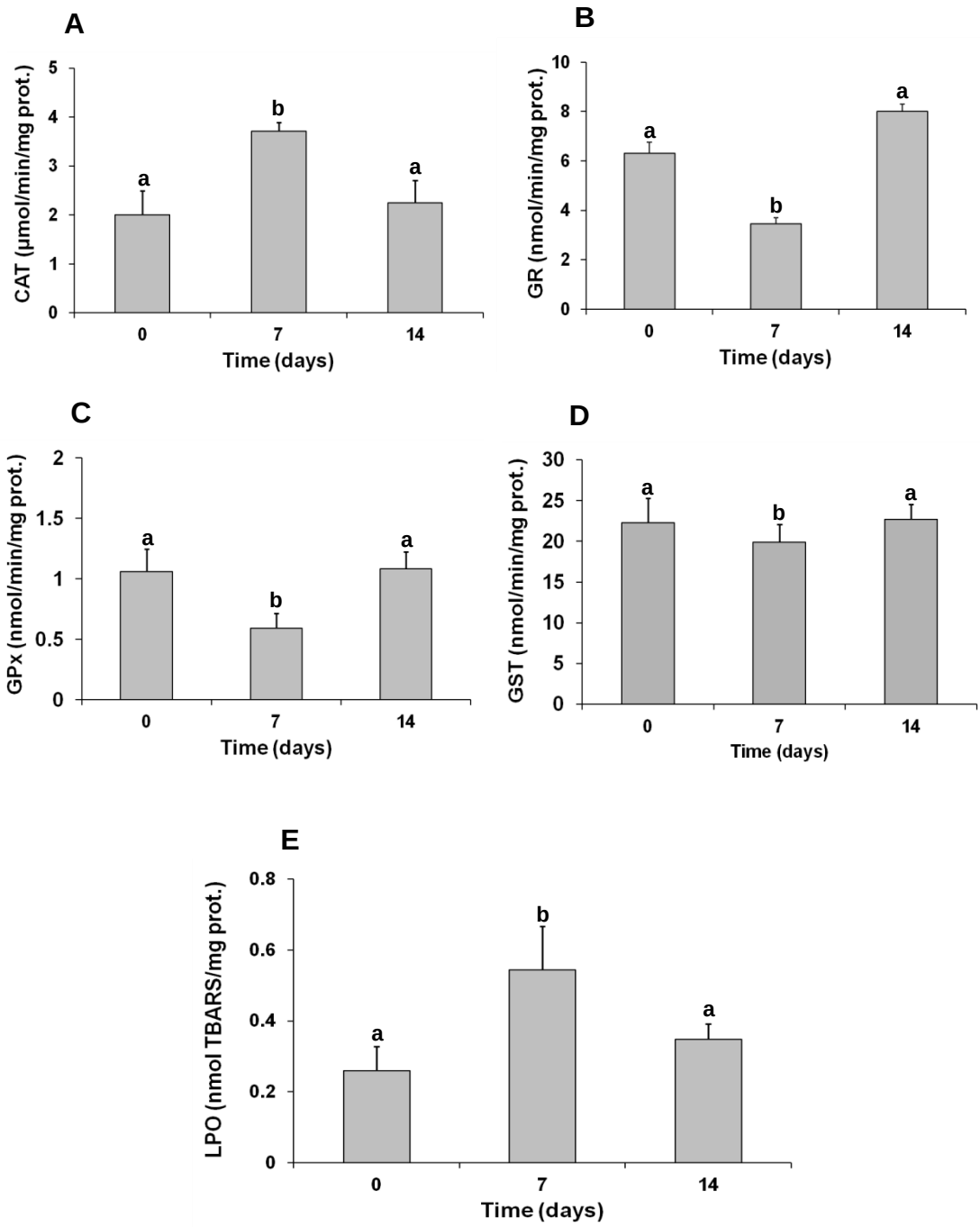

Fig. 3. A - Activity of catalase (CAT), B - Activity of glutathione reductase (GR), C - Activity of glutathione peroxidase (GPx), D - Activity of glutathione *S*-transferases (GST) and E - Lipid peroxidation (LPO) levels of *Corbicula fluminea* determined after arrival to laboratory (day 0) and after acclimation to laboratory conditions for 7 and 14 days. The values are the mean ± standard error of the mean of 9 organisms. Significant differences between treatments are identified by different letters above the bars (one-way ANOVA and the Tukey's test, $p \leq 0.05$).

Temperature is undoubtedly one of the most important factors that influence biological processes, including filtration rates and assimilation efficiency (Widdows and Bayne 1971; Sokolova and Pörtner, 2001). The dependence of enzymatic rates on temperature (Elias *et al*., 2014) can explain, at least in part, the increase observed in ChE activity in *C. fluminea* after 7 days of acclimation). Alterations in ChE activity related with temperature have also been described for other species. For instance, Pfeifer *et al*. (2005) reported maximum acetylcholinesterase activities in *Mytilus* spp. sampled during summer, positively correlated with increased water temperatures. Likewise, the influence of increasing summer temperatures on acetylcholinesterase activity was reported for *M. galloprovincialis* inhabiting a natural lagoon (Kamel *et al*., 2014). In a field study with *Morone saxatilis* a relationship between increased brain acetylcholinesterase activity and increased temperature (Durieux *et al*., 2011) was found.

Foot IDH activity was significantly increased after 7 days of acclimation (Fig. 2B), suggesting alterations in the aerobic pathways of energy production, probably related to an adjustment of the overall components of energetic metabolism to acclimation conditions (Fig. 2B). The dependence on temperature of some metabolic enzymes, including IDH, was previously observed. For instance juveniles of *Dicentrarchus labrax* maintained at 25 °C in laboratory conditions had higher IDH activity than those maintained at 18 °C (Almeida *et al*., 2015).

CAT, one of the first-line enzymes of the antioxidant system, and LPO levels were also found to be increased after 7 days of acclimation, indicating oxidative stress and oxidative damage (Figs. 3A and 3E). However, no direct relation between CAT activity and levels of LPO and temperature was found in *C. fluminea* in a two-year seasonal study with *C. fluminea* (Vidal *et al*., 2002b) probably because multiple abiotic conditions on the field may have contributed for the differences observed in these biomarkers.

Contrary to CAT activity, GR and GPx activities were significantly decreased (45% and 56%, respectively) at T_7 in relation to T_0 (Figs. 3B and 3C).

Regarding GST activity, it was also found inhibited (11%, Fig. 3D) but the decrease was notably lower than those observed for GR and GPx activities. Similarly, Quintaneiro *et al*. (2008) observed that GST of *Pomatochistus microps* collected in a reference site of M-est had a slower response to acclimation to laboratory conditions than AChE, suggesting that GST may be more stable towards environmental changes.

After 14 days of acclimation, the biomarkers levels were not significantly different from those determined at T_0. (Figs. 2 and 3), indicating recovery of the animals. Thus, 14 days of acclimation is an adequate acclimation period before using *C. fluminea* from wild

populations in bioassays based on the tested biomarkers for this specific population (ChE, IDH, CAT, GR GPx and GST activities, and LPO levels).

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CHAPTER III

Comparative sensitivity of European native (*Anodonta anatina*) and exotic (*Corbicula fluminea*) bivalves to mercury

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Abstract

Pollution is believed to be an important factor modulating the competition between exotic invasive bivalves and their native competitors. Thus, the objective of the present study was to compare the sensitivity of the European native *Anodonta anatina* and the exotic invasive species *Corbicula fluminea* to mercury, a ubiquitous environmental contaminant of high concern. In laboratory acute bioassays, adult organisms of both species were exposed independently to mercury for 96 h (31–500 µg/L). The criteria indicative of toxicity were mortality and biomarkers of oxidative stress and damage, neurotoxicity, and energy production changes. Mercury induced mortality in *A. anatina* (72 h-LC_{10} and 72 h-LC_{50} of 14.0 µg/L and 49.6 µg/L, respectively) but not in *C. fluminea*. The ability of *C. fluminea* to maintaining the shell closed for considerable periods of time when exposed to high concentrations of mercury and the effective activation (up to 63 µg/L) of mechanisms against the oxidative stress caused by mercury may have contributed to its relatively low sensitivity. In the range of concentrations tested, mercury had no significant effects on the other parameters analysed in *C. fluminea*. Overall, the findings of the present study, suggest that in real scenarios of competition between *C. fluminea* and *A. anatina* populations, the presence of mercury may modulate the process, acting in favour of the exotic species because it is less sensitive to this environmental contaminant than the native bivalve. The results of the present study highlight the need of further investigation on the effects of mercury on the competition between exotic invasive species and their native competitors, especially the effects potentially induced by long-term exposure to low concentrations of this metal, the mechanisms involved in the tolerance to mercury-induced stress, and the potential post-exposure recovery of both exotic invasive and native bivalves. This knowledge is most important for environmental management and assessment of the risks associated with the consumption of bivalves by humans.

Keywords: *Anodonta anatina*, *Corbicula fluminea*, Toxicity, Mercury, Biomarkers, Invasive alien species

3.1. Introduction

Biological invasions are a major threat to biodiversity conservation and to environmental and human health. Several species, including some pathogens to humans, have spread worldwide, and the success of new invasions is expected to further increase as the result of global climate changes (Bellard *et al*., 2013). In freshwater ecosystems of Europe, North America and other regions, *Corbicula fluminea* is among the most concerning exotic invasive species due to the negative ecological and economic impacts that its invasions may cause. For example, in ecosystems where *C. fluminea* population reaches a high abundance, the phytoplankton community may be considerably reduced due to the high filtration rates of this invasive species, decreasing the food availability for zooplankton and other first consumers, including bivalves (Mcmahon, 2002). Moreover, under these conditions, *C. fluminea* feeding and excretion may considerably alter nutrients cycling (Vaughn and Hakenkamp, 2001). Massive mortality events occurring regularly in *C. fluminea* populations may also have considerable adverse impacts on water quality and ecosystem function (Ilarri *et al*., 2011; Oliveira *et al*., 2015a). Regarding negative economic impacts of *C. fluminea*, the most important ones are frequently caused by its biofouling activity (Pimentel *et al*., 2005; Rosa *et al*., 2011). In several ecosystems colonised by *C. fluminea*, the introduction of this species is believed to be one of the causes contributing to the decline of native populations of unionid bivalves; one of the most endangered faunal groups in Europe and North America (Williams *et al*., 1993). Several environmental factors may influence the competition between *C. fluminea* and its native competitors, including habitat loss and degradation, extreme climate events resulting in draughts and floods, among others (Sousa *et al*., 2008f; Ilarri *et al*., 2011; Gallardo and Aldridge, 2013). These factors often act in favour of *C. fluminea* because, in general, it recovers more rapidly from negative impacts than native bivalves, mainly due to its higher reproduction capability (Mcmahon, 2002). Another factor that may influence the competition between *C. fluminea* and native bivalves is the presence of environmental contaminants because distinct species generally have different levels of sensitivity to chemical stress (Doherty and Cherry, 1988). Considering a simple scenario of two populations with distinct sensitivities to a chemical stressor in competition for limited resources, the more tolerant one is expected to gradually increase its fitness, potentially leading to the extirpation of the most sensitive one. Therefore, if the most tolerant species is a bioinvasor, the presence of an environmental contaminant may act in its benefit (Piola and Johnston, 2008). *C. fluminea* was found to be more tolerant to several common environmental contaminants than other freshwater bivalves. For example, the 96 h median lethal concentrations (LC_{50}) of arsenic, zinc and cadmium to adult *C. fluminea*

were 20.74 mg/L, 6.04 mg/L and 32 mg/L, respectively, while the corresponding values to adult *Lammelidens corrianus*, *L. consobrinus*, and *Pisidium casertanum* were 1.34 mg/L, 1.68 mg/L and 1.37 mg/L, respectively (Rodgers *et al*., 1980; Giesy *et al*., 1983; Mackie, 1989; Liao *et al*., 2008; Bhamre *et al*., 2010; Gulbhile and Zambare, 2013). However, *C. fluminea* is more sensitive to ammonia (96 h-LC_{50} = 13.96 mg/L) than *Pyganodon grandis* (96 h-LC_{50} = 25.13 mg/L); to lead (96 h-LC_{50} = 1.02 mg/L) than *P. casertanum* (96 h-LC_{50} = 23.50 mg/L); and to pentachlorophenol (96 h-LC_{50} = 0.23 mg/L) than *Sphaerium novazelandiae* (96 h-LC_{50} = 243 mg/L) (Mackie, 1989; Scheller, 1997; Hickey and Martin, 1999; Labrot *et al*., 1999; Jin *et al*., 2012). Moreover, in a field transplantation study carried out in the Ebro River system, *C. fluminea* was found to be less tolerant to environmental contamination (including mercury) than the native species *Psilunio littoralis* (Faria *et al*., 2010). Therefore, more research on this topic is needed to understand the effects that environmental contamination may have on the competition between *C. fluminea* and native species of high conservational interest. This knowledge is most important to support scientifically based management actions.

The objective of this study was to compare the sensitivity of *C. fluminea* and *Anodonta anatina* to acute mercury exposure. *A. anatina* was selected as the native competitor model because unionid bivalves have high conservational interest, and the presence of *C. fluminea* has been reported to contribute to the decline of their populations (Mouthon and Daufresne, 2010). Mercury was selected as the test substance for this study because: (i) it is an ubiquitous environmental contaminant of global concern due to the negative effects on human and environmental health that it may cause (UNEP, 2013); (ii) it is known to be accumulated by several bivalves including *C. fluminea* (Ravera *et al*., 2009; Neufeld, 2010; Waykar and Shinde, 2011); (iii) it causes mortality to freshwater bivalves at concentrations in the low ppm range (Sivaramakrishna *et al*., 1991) or lower ones (Keller and Zam, 1991); (iv) it is commonly found in freshwater ecosystems where *C. fluminea* and *A. anatina* coexist (Bódis *et al*., 2014b; Comero *et al*., 2014); and (v) more knowledge on its toxic effects are needed to improve environmental and human health risk assessment and safety measures.

A multi-parameters approach was selected to assess the toxicity of mercury to test organisms, including mortality and sub-individual biomarkers allowing to assess neurotoxicity, oxidative stress and damage, and energy production alterations because these functions are crucial for individual fitness (Luís and Guilhermino, 2012). The activity of cholinesterase enzymes (ChE) was selected as neurotoxicity biomarker mainly because it has been widely used for this effect, including in *C. fluminea* (*e.g.* Oliveira *et al*., 2015a), and mercury is known to inhibit the activity of these enzymes in some species (Suresh *et al*., 1992; Elumalai *et al*., 2007). The antioxidant enzymes glutathione

reductase (GR), glutathione *S*-transferases (GST), catalase (CAT), glutathione peroxidase (GPx) and lipid peroxidation (LPO) levels were selected because, as a whole, they allow assessing antioxidant responses and lipid oxidative damage induced by environmental contaminants (Lima *et al*., 2007; Almeida *et al*., 2014), including mercury (Vieira *et al*., 2009). The enzymes NADP-dependent isocitrate dehydrogenase (IDH) and octopine dehydrogenase (ODH) are most important in the pathways of energy production in molluscs (Baldwin and Opie, 1978; Ivanina *et al*., 2008), and IDH is also crucial to maintain the cellular redox status (Lee *et al*., 2002). Thus, they have been widely used as biomarkers of effects on these processes (*e.g.* Troncoso *et al*., 2000; Lima *et al*., 2007; Oliveira *et al*., 2015a) and were selected for the present study.

3.2. Material and methods

3.2.1. Chemicals

Mercury (II) chloride (CAS no. 7487-94-7) (≥ 99.5% purity) was purchased from Sigma-Aldrich (Germany). All the other chemicals used were of analytical grade and purchased from Sigma-Aldrich (Germany) or Merck (Germany). The Bradford reagent was purchased from Bio-Rad (Germany).

3.2.2. Collection and laboratory maintenance of organisms

Adult specimens of *C. fluminea* with approximately 25–30 mm (anterior-posterior shell length) were collected in the Minho River upper estuary, Northwest of Portugal (~42°30'22.51''N 8°32'22.51''W) with an adapted rake. They were immediately transported to the laboratory in thermally isolated boxes with water from their collection site. Despite having punctual sources of contamination, the Minho River estuary is considered a low polluted system (Santos *et al*., 2013). The population of *A. anatina* in this estuary has been decreasing over the last decade and has now a very low density and biomass (Sousa *et al*., 2008b). Thus, due to conservational reasons, the specimens of *A. anatina* used in the present study were not collected in the Minho River estuary. They were collected in a pristine area of the Tâmega River (~41°24'48.6''N 7°57'53.8''W) having a population with a high number of individuals (Sousa *et al*., 2012). Native bivalves with approximately 90–116 mm (anterior-posterior shell length) were handpicked using the snorkelling technique. Organisms from both species were acclimated to laboratory conditions for 14 days, in a temperature (16 ± 1° C) and photoperiod (16 h light (L): 8 h dark (D)) controlled room. They were maintained in plastic boxes filled with 16 L of dechlorinated tap water (DTW). Each box had 16 bivalves of the same species. Water was renewed three times per week and continuous aeration was provided. Bivalves were

fed with a mixture of *Chlorella vulgaris* and *Chlamydomonas reinhardtii* (50%: 50% cells/cells) in a total ratio of 8 × 10^5 algae cells/day/bivalve (from algae laboratorial cultures). This type of food was selected because it provides a suitable nutrition for both species (Foe and Knight, 1986; Lima *et al*., 2006). Water temperature, pH and conductivity were monitored at the beginning of the bioassays and at the time of clean medium renewal using a Multi 340i/Set Wissenschaftlich-Technische Werkstätten multiparametric probe (Germany). Water dissolved oxygen was measured using an Oxi 320/Set Wissenschaftlich-Technische Werkstätten oxygen probe (Germany).

3.2.3. Mercury bioassay

3.2.3.1. Experimental design and exposure conditions

The 96 h bioassays were carried out in a temperature (16 ± 1 °C) and photoperiod (16 h L: 8 h D) controlled room. The effect criteria were mortality and the following biomarkers: ChE activity as indicative of neurotoxicity; the activity of the enzymes IDH and ODH involved in the pathways of energy production; the activity of GR, GST, CAT and GPx, which are part of antioxidant defences; the LPO levels as marker of lipid oxidative damage. On the first day of the bioassays, a stock solution of mercury (II) chloride was prepared in DTW with a concentration of 1 mg/L of mercury. From this solution, 5 mercury treatments were prepared by serial dilution of the stock solution in DTW, which was used as test media: 31, 63, 125, 250 and 500 µg/L (mercury concentrations). The control treatment was DTW only. Glass beakers (2 L) filled with 1.6 L of test medium, with continuous aeration were used. After acclimation in the conditions previously indicated (section 3.2.2), *C. fluminea* (29 ± 2.4 mm) and *A. anatina* (106 ± 9.6 mm) specimens were randomly selected and moved to clean media Food was stopped 48 h before the bioassays. On the first day of the bioassays, clams were randomly distributed to different treatments, with 9 *C. fluminea* and 3 *A. anatina* specimens per treatment. Due to the conservational interest of the species, only 3 specimens of *A. anatina* were used per treatment. All the organisms were individually exposed *(i.e.* 1 organism per test beaker) for 96 h, no food was provided during the bioassays, and test media were renewed at each 48 h interval. Organisms were observed as much as possible during the day. Dead organisms were removed as soon as noticed and mortality was recorded at each 24 h. Water temperature, pH, and conductivity were measured at the beginning of the bioassay and then at each 48 h interval in new and old medium using a Multi 340i/Set Wissenschaftlich-Technische Werkstätten multiparametric probe (Germany). Water dissolved oxygen was measured at the same time using an Oxi 320/Set Wissenschaftlich-Technische Werkstätten oxygen probe (Germany).

3.2.3.2. Biomarkers determination

The biomarkers were determined in *C. fluminea* only, due to the high mortality recorded in *A. anatina* exposed to mercury. At the end of the bioassay, tissues were isolated from each clam and stored at −80 °C until further analysis. The adductor muscle was used for ChE determinations; the foot was used for IDH and ODH analyses; and gills were used for GR, GST, CAT, GPx, and LPO determinations. A piece of adductor muscle (0.25 mg) was isolated on ice and put in 1 mL of cold potassium phosphate buffer (0.1M, pH = 7.2). From the foot, two pieces (about 0.30 mg each) were isolated on ice: one was put in 1 mL of cold tris(hydroxymethyl)aminomethane buffer (Tris buffer) (50 mM, pH = 7.8) and the other piece was put in 1 mL of cold Tris buffer (20 mM, pH = 7.5) with ethylenediaminetetraacetic acid disodium salt dehydrate 1 mM and DL-1,4-Dithiothreitol 1 mM. Gill tissue was put in cold potassium phosphate buffer (0.1 M, pH = 7.4) in a 1:10 ratio (weight/volume).

On the day of the analysis, sample homogenates were prepared (in the buffers in which they were frozen) on ice using an Ystral GmbH d-7801 homogenizer (Dottingen, Germany). Adductor muscle and foot samples were homogenized for 1 min and then centrifuged at 3300 ×*g* for 3 min at 4 °C. After homogenization of gills for 1 min, the homogenate was divided in two parts: gill homogenate A and gill homogenate B. Gill homogenate B was stored at −80 ºC to further determination of LPO levels. Gill homogenate A was centrifuged at 10000 ×*g* for 20 min at 4 °C. A Sigma Laboratory Centrifuge, model 3K30 (Germany) was used for all the centrifugations. All the supernatants were carefully collected, maintained on ice, and their protein content was determined by the Bradford method (Bradford, 1976) adapted to microplate (Frasco and Guilhermino, 2002) using bovine *γ*-globulin as protein standard. Sample protein content was then standardized to 1 mg/mL for ChE, IDH and ODH analyses, and to 4 mg/mL for the determination of antioxidant enzymes.

The determination of ChE activity was done according to the Ellman's technique (Ellman *et al*., 1961) adapted to microplate (Guilhermino *et al*., 1996), using 0.05 mL of adductor muscle supernatant. The hydrolysis of the substrate acetylthiocholine was indirectly determined through the production of the anion 5-thionitro-benzoic acid at 412 nm, for 5 min. IDH activity was determined in 0.05 mL of foot supernatant through the measurement of NADPH increase at 340 nm for 3 min, according to Ellis and Goldberg (1971) adapted to microplate by Lima *et al*. (2007). ODH activity was determined in 0.05 mL of foot supernatant, through the consumption of pyruvate due to nicotinamide adenine dinucleotide (NADH) oxidation at 340 nm for 3 min, following the method suggested by Livingstone *et al*. (1990) with small adaptations (Lima *et al*., 2007). GR activity was

determined in 0.1 mL of gill supernatant, according to Carlberg and Mannervik (1985), following the decrease in nicotinamide adenine dinucleotide phosphate (NADPH) for 1 min at 340 nm. GST activity was measured in 0.05 mL of gill supernatant, based on the reaction of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene. The production of dinitrophenyl thioether was monitored at 340 nm for 1 min, according to Habig *et al*. (1974), adapted to microplate by Frasco and Guilhermino (2002). CAT activity was determined in 0.05 mL of gill supernatant, following the degradation of hydrogen peroxide (H_2O_2) at 240 nm for 1 min, according to Clairborne (1985). GPx activity was determined in 0.09 mL of gill supernatant, according to Mohandas *et al*. (1984), using H_2O_2 as substrate, and monitoring the NADPH decrease at 340 nm, for 1 min. In all the enzymatic determinations, the slope of the linear part of the reaction curve was used, and the enzymatic activities were expressed as nanomoles of substrate hydrolyzed per minute per mg of protein (nmol/min/mg protein) with the exception of CAT activity that was expressed in micromoles of substrate hydrolyzed per minute per mg of protein (µmol/min/mg protein). LPO levels were determined in 0.2 mL of gill homogenate B, through the determination of thiobarbituric acid-reactive substances (TBARS) at 535 nm, according to Ohkawa *et al*. (1979) and Bird and Draper (1984), with adaptations (Filho *et al*. 2001; Torres *et al*. 2002). They were expressed in nanomoles of TBARS per mg of protein (nmol TBARS/mg protein). Sample protein content was determined at the end of enzymatic determinations and quantification of LPO levels as previous indicated. All the biomarkers analysis and protein determinations were carried out at 25 ºC in a Spectramax® M2 spectrophotometer (Molecular Devices, U.S.A.).

3.2.4. Statistical analysis

At the end of the bioassays, the percentages of *A. anatina* mortality were determined, transformed to probit units and plotted against the corresponding log transformed mercury concentrations. The LC_{10}, LC_{20} and LC_{50} at 72 h, and the median lethal time (LT_{50}) of organisms exposed to 125 µg/L of mercury were calculated from the toxicity curve. The LT_{50} values were calculated for this concentration only because in the other concentrations the percentages of mortality recorded over time did not allow an adequate fitting of the probit model. For each biomarker determined in *C. fluminea*, data were checked for distribution, normality and homogeneity of variances using the Shapiro-Wilk and Levene's tests, respectively. A logarithmic transformation was used when data did not fulfil the assumptions of normality or homogeneity of variances. Then, each set of data was analyzed by one-way Analysis of Variance (ANOVA). When significant differences were found, a Tukey's test was used to identify significantly different treatments.

The significance level was 0.05 in all the analyses performed, and the software IBM SPSS Statistics package (U.S.A), version 22.0, was used.

3.3. Results and discussion

3.3.1. Comparative sensitivity to mercury

Water mercury concentrations comparable to the lowest concentration of mercury tested in the present study (31 µg/L) have been found in heavy contaminated sites. For example, concentrations of mercury up to 35 µg/L were found in the water of natural ecosystems close to mining areas in South Eastern U.S.A. (Mastrine *et al*., 1999). Thus, at least the mercury concentration of 31 µg/L is ecologically relevant. The other concentrations tested were selected to investigate the effects of mercury on *C. fluminea* and *A. anatina* with no concerns regarding their ecological relevance.

In the range of concentrations tested, mercury induced mortality on *A. anatina* (Table 3), reaching 100% after 24 h of exposure to 500 µg/L and 67% after 96 h of exposure to the lowest concentration tested (31 µg/L). The 72 h-LC_{10}, the 72 h-LC_{20} and the 72 h-LC_{50} of mercury to *A. anatina* determined in the present study were 14.0 µg/L (95% CI: 0–35.2 µg/L), 21.6 µg/L (95% CI: 0.006–46.6 µg/L), and 49.6 µg/L (95% CI: 21.0–99.6 µg/L), respectively. The LT_{50} determined for the concentration of 125 µg/L was 60 h (95% CI: 48.9–71.3 h).

Table 3. Percentages of mortality induced by different concentrations of mercury on *Anodonta anatina* over 96 hours of exposure through test medium. For ethical reasons, only 3 specimens were used per treatment.

	Mortality recorded along time (hours)			
Mercury concentrations (µg/L)	24	48	72	96
0	0	0	0	0
31	0	0	33	67
63	0	33	67	67
125	0	33	67	100
250	67	67	100	100
500	100	100	100	100

No mortality was recorded in *C. fluminea* after 96 h of exposure to mercury concentrations up to 500 µg/L. These results indicate that the native species was more sensitive to mercury induced acute stress than *C. fluminea*. Several processes may contribute to the differences of sensitivity between the two species found in the present study, including toxicant avoidance behaviour of *C. fluminea*, and differences in the mechanisms of uptake, biotransformation, and elimination of mercury between the two species. During the bioassay, *C. fluminea* maintained the shell closed for considerable periods of time, likely reducing the exposure to mercury, as suggested in a previous study with this species also exposed to mercury (Tran *et al*., 2007). Thus, this behaviour that was not observed in *A. anatina* during our experiments, may have contributed to the lower sensitivity of *C. fluminea* to mercury relatively to *A. anatina*. Regarding other processes, such as mercury uptake and elimination, they were not investigated in the scope of this study. In relation to mechanisms of toxicity and responses to chemical stress that were investigated in *C. fluminea*, the corresponding biomarkers could not be determined in the native species due to the high mortality recorded.

In a previous study carried out by Tran *et al*. (2007), no mortality was recorded in *C. fluminea* exposed for 5 h to 300 µg/L of mercury, thus providing support to the relative low sensitivity of this bivalve to mercury found in the present work. Moreover, the 48 h-LC_{50} of mercury to *L. consobrinus* was 1860 µg/L and the 96 h-LC_{50} of this metal to *L. marginalis* was 10000 µg/L (Hameed and Raj, 1989; Bhamre *et al*. 2010), whereas the 72 h-LC_{50} of mercury to *A. anatina* determined in the present study was 49.6 µg/L. Therefore, despite the differences in the experimental conditions used in distinct studies, these findings suggest important differences of sensitivity to mercury among freshwater bivalve species.

When two populations of distinct species with different sensitivities to chemicals are under competition in contaminated environments, the most tolerant one may gradually increase its fitness and it may eventually eliminate the most sensitive one (Lajtner and Crnčan, 2011). Therefore, if the most tolerant species is a bioinvasor, the presence of environmental contaminants may be an important factor contributing to the success of the invasion, as previously suggested (Karatayev *et al*. 2009). Thus, despite the high concentrations of mercury tested in the present study, the difference in sensitivity between *C. fluminea* and *A. anatina* found suggests that in competition scenarios, the presence of this metal may benefit the exotic invasive species relatively to the native one.

3.3.2. Effects of mercury on *C. fluminea* biomarkers

The effects of mercury on *C. fluminea* biomarkers are shown in Figs. 4 and 5, and the results of the corresponding statistical analyses are indicated in Table 4. No significant effects of mercury on ChE activity were found (Fig. 4A, Table 4). Thus, in the range of concentrations tested, mercury was not able to impair the cholinergic transmission through ChE inhibition in *C. fluminea*. The *in vivo* effects of mercury on ChE activity of several species have been previously investigated. For example, a significant inhibition of head and muscle ChE activity was found in juveniles of the common goby (*Pomatoschistus microps*) after 96 h of exposure to concentrations of mercury in the water equal or higher than 3.125 µg/L (Vieira *et al*., 2009); a significant decrease of ChE activity was also found in the red swamp crayfish, *Procambarus clarkii*, exposed for 24 h to 200 µg/L of mercury through the water (Devi and Fingerman, 1995); and a significant inhibition of eye ChE activity (EC_{50} = 235 µg/L) was found in *Carcinus maenas* exposed for 96 h to mercury through the water (Elumalai *et al*., 2007). However, no significant effects of mercury on ChE activity after *in vivo* exposure were also reported, for example in the common prawn (*Palaemon serratus*) exposed to mercury concentrations of 1 and 5 mM for up 7 days (Frasco *et al*., 2008).

Table 4. Results of the one-way ANOVA carried out with the data of each biomarker to compare different treatments. ChE - Cholinesterase enzymes activity; IDH - NADP-dependent isocitrate dehydrogenase activity; ODH - Octopine dehydrogenase activity; GR - Glutathione reductase activity; GST - Glutathione *S*-transferases activity; CAT - Catalase activity; GPx - Glutathione peroxidase activity; LPO - Lipid peroxidation levels; df - Degrees of freedom.

Biomarker	df	F	p
ChE	(5, 48)	0.649	0.664
IDH	(5, 42)	24.97	0.000
ODH	(5, 44)	0.881	0.548
GR	(5, 40)	3.220	0.015
GST	(5, 46)	6.032	0.000
CAT	(5, 42)	5.794	0.000
GPx	(5, 45)	2.340	0.015
LPO	(5, 48)	4.492	0.020

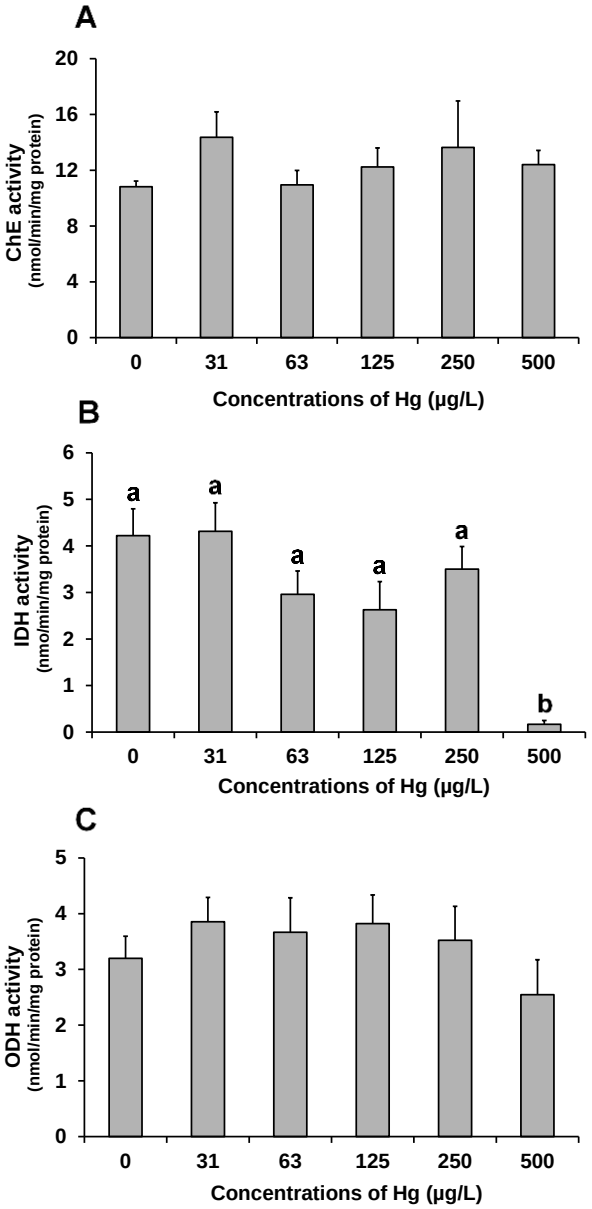

Fig. 4. Effects of mercury on biomarkers of neurotoxicity and energetic metabolism of *Corbicula fluminea*. The values are the mean of 9 clams with the corresponding S.E.M. bars. A - Activity of cholinesterase enzymes (ChE) determined in the adductor muscle. B - Activity of NADP-dependent isocitrate dehydrogenase (IDH) determined in the foot. C - Activity of octopine dehydrogenase (ODH) determined in the foot. Significant differences between treatments are identified by different letters above the bars (one-way ANOVA and the Tukey's test, $p \leq 0.05$).

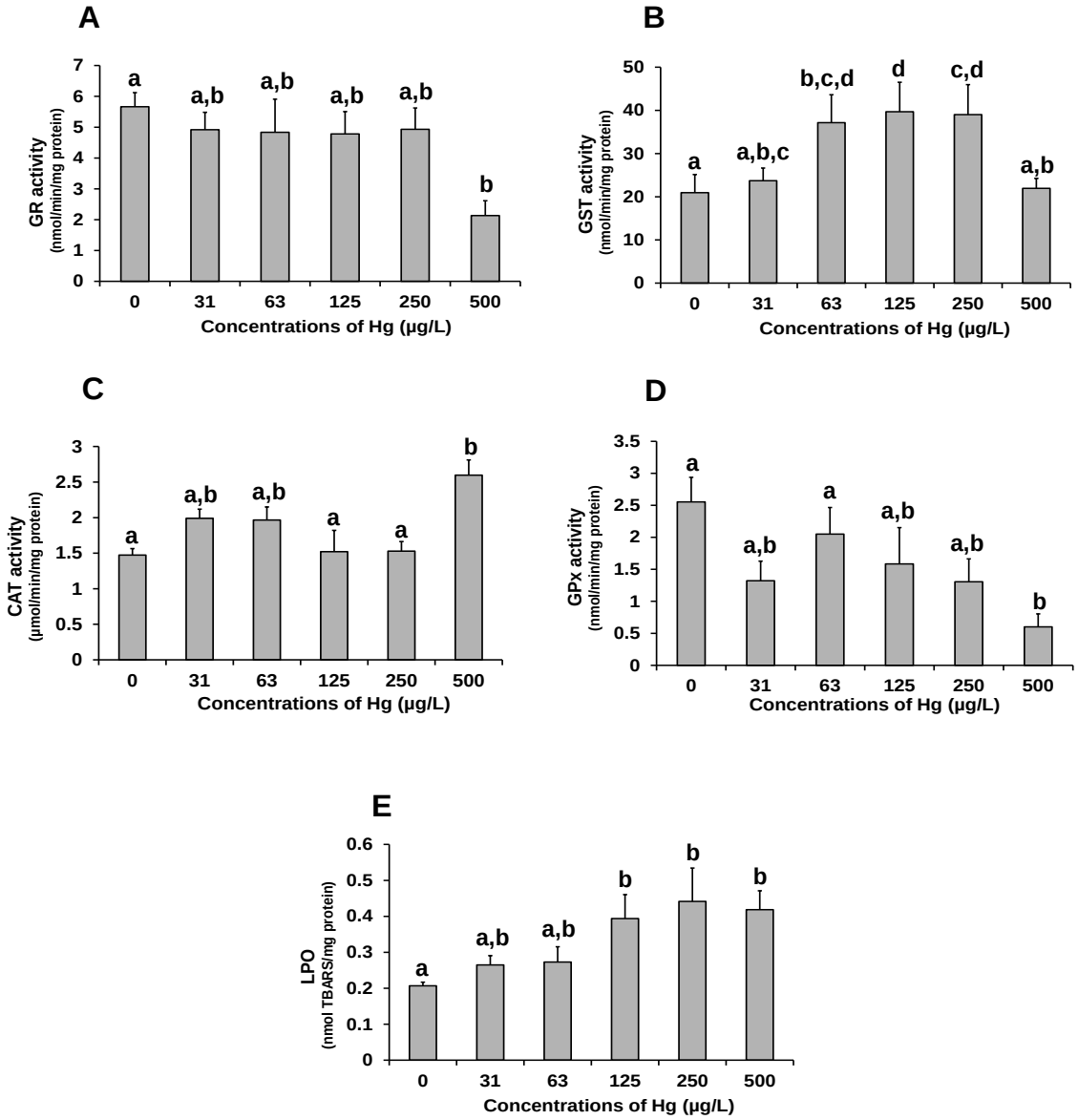

Fig. 5. Effects of mercury on biomarkers of oxidative stress and damage of *Corbicula fluminea*. The values are the mean of 9 bivalves with the corresponding S.E.M. bars. A - activity of glutathione reductase (GR), B - activity of glutathione *S*-transferases (GST), C - activity of catalase (CAT), D - activity of glutathione peroxidase (GPx) and E - levels of lipid peroxidation (LPO). Significant differences between treatments are identified by different letters above the bars (one-way ANOVA and the Tukey's test, $p \leq 0.05$).

As shown by *in vitro* studies, mercury is able to inhibit the ChE activity of several species while having no significant effects in others. Kopecka-Pilarczyk (2010) reported 50% of ChE inhibition in tissues of *Mytilus trossulus* after incubation with concentrations equal or higher than 4×10^{-3} g/L. In another study, mercury concentrations of 1–10 µM significantly inhibited the activity of *Torperdo californica* ChE, but had no significant effects of the enzymes of *Drosophila melanogaster* and *Electrophorus electricus* in this range of concentrations (Frasco *et al*., 2007). The presence of a free sensitive sulfhydryl group in the enzyme seems to be important to the kinetics of the reaction between the enzyme and mercury, determining the concentration of mercury needed to inhibit the enzyme activity (Frasco *et al*., 2007). Thus, among other possibilities, it can be hypothesized that the ChE(s) of *C. fluminea* adductor muscle do not have a free sensitive sulfhydryl group which may be important in the reaction between the metal and the enzyme(s).

Regarding the enzymes involved in the pathways of energy production, no significant effects of mercury on *C. fluminea* ODH activity were found (Fig. 4C, Table 4). However, the activity of *C. fluminea* IDH was significantly reduced after 96 h of exposure to 500 µg/L of mercury (Fig. 4B, Table 4.). Since IDH participates in the aerobic energy production, the reduction of oxygen due to the shell closing behaviour under heavy mercury exposure, may have contributed to the decrease of the enzymatic activity. Also, mercury is known to interact with the sulfhydryl groups of enzymes, inhibiting their catalytic activity (Bridges and Zalups, 2005). Thus, the IDH inhibition found in the present study may also be due to this effect. Independently of the process(s) involved, the inhibition of IDH activity potentially results in a decrease of NADP reduction to NADPH (Rodriguez *et al*., 2004). Since NADPH is a GR co-factor, its decrease likely causes a reduction of this enzyme activity. Since GR regenerates glutathione, the inhibition of this enzyme may lead to glutathione depletion, and to inhibition of GPx and GST that require glutathione to function (Cooper and Kristal, 1997). This may have happened in the clams exposed to 500 µg/L of mercury, as suggested by the significant decrease of IDH, GR, and GPx activities (Figs. 4B, 5A and 5D), and the return of GST activity to levels similar to those of the control group (Fig. 5B) after an induction of this enzyme activity at lower mercury concentrations (63–250 µg/L).

The changes in the activity of *C. fluminea* antioxidant enzymes shown in Fig. 5 indicate that, in the range of concentrations tested, mercury induces oxidative stress in this species. A significant induction of GST activity was found in clams exposed to 63 µg/L, with no significant alterations in the activity of the other antioxidant enzymes (Fig. 5A–D). This finding suggests that GST induction is the first response of *C. fluminea* to mercury induced oxidative stress. GST induction was maintained up to 250 µg/L and the activity of the enzyme returned to values not significantly different from those determined in the

control group in clams exposed to 500 µg/L (Fig. 5B), thus displaying the bell shape pattern of this enzyme that has been found in other species exposed to different environmental contaminants (Vieira *et al*., 2009; Kamel *et al*., 2012). Under exposure to 500 µg/L of mercury, CAT was significantly induced (Fig. 5C) possibly to compensate the significant inhibition of GR and GPx, and the changes of GST activity likely caused by IDH inhibition as previously discussed. The results of Fig. 5E and Table 4 also show a significant increase of LPO levels (~2 folds) in *C. fluminea* exposed to mercury concentrations equal or higher than 125 µg/L indicating lipid peroxidation damage. Thus, the activation of GST activity at concentrations between 63 and 250 µg/L, and the increase of CAT activity at 500 µg/L were not enough to overcome the oxidative stress caused by mercury, and lipid peroxidation occurred.

Mercury exposure is known to cause oxidative stress and damage in other species, including bivalves such as *M. edulis* (Geret *et al*., 2002). Despite the methodological differences, evidences from the literature and the present study suggest some differences in the response to oxidative stress caused by mercury among distinct species. For example, a reduction in the activity of *Clamys farreri* CAT and GPx enzymes under mercury exposure was found (Zhang *et al*., 2010). Thus, the inhibition of *C. fluminea* GPx caused by the highest concentration of mercury tested (Fig. 5D) is in agreement with the effects of this metal in *C. farreri*. However, contrary to the inhibition caused by mercury on *C. farreri* CAT activity (Zhang *et al*., 2010), an induction of CAT activity was found in *C. fluminea* exposed to 500 µg/L of mercury (Fig. 5C, Table 4), suggesting some differences in the response to the mercury induced oxidative stress between the two species. As in *C. fluminea*, an induction of CAT and GST activities in response to mercury exposure was found in the bivalve *Perna viridis* (Verlecar *et al*., 2008) and the prawn *Macrobrachium malcolmsonii* (Yamuna *et al*., 2012).

3.4. Conclusions

In summary, mercury (31–500 µg/L) induced a high mortality in *A. anatina* (native in Europe), with 72 h LC_{10}, LC_{20} and LC_{50} of 14.0 µg/L, 21.6 µg/L and 49.6 µg/L, respectively. No mortality was recorded in *C. fluminea* (exotic invasive species in Europe) after 96 h of exposure up to 500 µg/L of mercury. These results indicate that the native species was more sensitive to mercury-induced acute stress than the exotic invasive species. The ability of *C. fluminea* to keep the shell closed during exposure to high concentrations of mercury may have contributed to the relatively low sensitivity found. In addition, *C. fluminea* was able to induce antioxidant defences preventing the occurrence of lipid peroxidation damage up to 63 µg/L of mercury. At higher mercury concentrations,

the capability of the antioxidant stress system seems to have been exceeded and lipid oxidative damage occurred (~2 folds of LPO levels increase); under exposure to 500 µg/L of mercury, changes in the anaerobic pathway of energy production may also have occurred, as suggested by the almost full inhibition of IDH activity, an enzyme that is also important for the antioxidant system. In the range of concentrations tested, mercury had no anticholinesterase effects in *C. fluminea*. Overall, the findings of the present study, suggest that in real scenarios of competition between *C. fluminea* and *A. anatina* populations, the presence of mercury may modulate the process, acting in favour of the exotic species because it is less sensitive to this environmental contaminant than the native one. The results of the present study highlight the need of further investigation on the effects of mercury on the competition between exotic invasive species and their native competitors, especially through long-term exposure at ecologically relevant concentrations of this metal. More studies are also needed to understand the mechanisms involved in the tolerance to mercury-induced stress, as well as in the potential post-exposure recovery of both exotic invasive and native bivalves. This knowledge is most important for environmental risk assessment and management, and to improve the human safety when consuming bivalves as food.

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CHAPTER IV

Toxicity of mercury and post-exposure recovery in *Corbicula fluminea*: neurotoxicity, oxidative stress and oxygen consumption

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Abstract

The toxicity of mercury to the invasive species *Corbicula fluminea* and the post-exposure recovery were investigated in relation to previous developmental exposure to distinct environmental conditions. Bivalves were collected in the estuaries of Minho River (M-est) and Lima River (L-est) that have several abiotic differences, including in environmental contamination, with the former being generally less contaminated. After 14 days of acclimation to laboratory conditions, two 14-day bioassays were performed simultaneously: one with bivalves from the M-est and the other with bivalves from the L-est. In each bioassay, the treatments were: dechlorinated tap water (clean medium) for 8 days, clean medium for 14 days, 31 µg/L of mercury for 8 days, 31 µg/L of mercury for 14 days and 31 µg/L of mercury for 8 days followed by 6 days in clean medium (post-exposure recovery). The effect criteria were the oxygen consumption rate (OCR), the activity of the enzymes cholinesterases (ChE), NADP-dependent isocitrate dehydrogenase (IDH), octopine dehydrogenase (ODH), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione *S*-transferases (GST), and lipid peroxidation (LPO) levels. Exposure to mercury for 8 days caused significant ($p \leq 0.05$) inhibition of GR activity in M-est bivalves, whereas no significant adverse effects were observed in L-est animals. Moreover, evidences of delayed toxicity caused by 8-day exposure to mercury in OCR, IDH activity and LPO levels were found in M-est individuals but not in those of the L-est. Exposure to mercury for 14 days caused significant ($p \leq 0.05$) depression of the OCR and of IDH activity in animals from both estuaries, indicating reduced individual fitness and hypoxia conditions. Moreover, oxidative stress and lipid peroxidation damage were observed in bivalves from the M-est exposed to mercury for 14 days but not in L-est animals. Differences in M-est and L-est environmental conditions to which animals were exposed in the wild likely contributed to the differences of sensitivity to mercury between M-est and L-est bivalves. The results of this study highlight the importance of investigating delayed toxicity, post-exposure recovery, and of taking into consideration the background contamination and other abiotic conditions of the original habitats when assessing the effects of environmental contaminants on animals from wild populations.

Keywords: *Corbicula fluminea*, Mercury, Oxygen consumption, Delayed toxicity, Oxidative stress

4.1. Introduction

Corbicula fluminea is a freshwater bivalve native to Asia, Africa and Australia (Sousa *et al*., 2008a) and a non-native invasive species in several other worldwide regions (Araujo *et al*., 1993; Ituarte, 1994; Munjiu and Shubernetski, 2010; Chainho *et al*., 2015). It is considered one of the 100 worst invasive species in Europe (DAISIE, 2018). In many colonized aquatic ecosystems, *C. fluminea* became the dominant species, contributed to the decline of native species, affected dramatically important ecological processes, and caused severe economic losses (Pimentel *et al*., 2005; Sousa *et al*., 2008a; Strayer, 2010; Rosa *et al*., 2011). In such ecosystems, the eradication of the species is very difficult and often economically unviable. Thus, the control and management of the populations, including taking advantage of the services that such populations may provide (*e.g.* water clearing, source of food to humans, use of the species to assess the environmental quality and as model organism to investigate the effects of pollutants) are the most adequate management options (Doherty, 1990; Vidal *et al*., 2002b; Chijimatsu *et al*., 2009).

Mercury is a widespread environmental contaminant of high concern mainly because is highly toxic to the wildlife and humans (Bernhoft, 2012; Bjørklund *et al*., 2017). It is listed as priority hazardous substance (*e.g.* EC, 2008b). It may be accumulated by several species including *C. fluminea* (Cairrão *et al*., 2007; Liu *et al*., 2014; Neufeld, 2010), and some of its organic forms (*e.g.* methylmercury) may be biomagnified in trophic chains (Lavoie *et al*., 2013; Cardoso *et al*., 2014) increasing the risk of exposure and toxic effects to top predators and humans. Therefore, and despite the high number of studies carried out on the subject, it is most important to continue the investigation on the toxic effects of mercury because the knowledge regarding the sub-lethal effects in different species is still limited. This work investigated the effects of exposure to mercury in *C. fluminea*, the post-exposure recovery, and the influence of previous exposure to different environmental conditions in these processes, using individual and sub-individual biomarkers.

Biomarkers were selected as effect criteria mainly because they are early-warning signs of adverse effects caused by chemical-induced stress and provide information on the mechanisms of toxicity involved (van der Oost *et al*., 2003). The oxygen consumption rate (OCR) is recognized as an important physiological parameter on evaluating the effect of toxicant stress (Calow, 1991; Martins *et al*., 2007) and has been used as a biomarker in several studies with bivalves exposed to mercury (Saliba and Vella, 1977; Mohan *et al*., 1986; Devi, 1996; St-Amand *et al*., 1999). The activity of cholinesterase enzymes (ChE) was selected as indicative of neurotoxicity because the impairment of cholinergic transmission may affect a wide range of functions at sub-individual and individual levels (Richetti *et al*., 2011). The activity of NADP-dependent isocitrate dehydrogenase (IDH)

and octopine dehydrogenase (ODH) provide valuable information on aerobic and anaerobic cellular energy production, respectively. The activity of the enzymes catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione *S*-transferases (GST) were selected because they are crucial defences against oxidative stress, and lipid peroxidation (LPO) levels were used as indicative of lipid oxidative damage. Moreover, these biomarkers have been widely used to assess the effects of environmental contaminants on aquatic organisms, including on *C. fluminea* (Graney and Giesy, 1988; Oliveira *et al*., 2015b; Dong *et al*., 2016).

C. fluminea was selected as test organism for this study mainly because: (i) it is an exotic invasive species in several regions of the world and therefore the use of individuals from wild populations of these areas generally has no negative conservation impacts; (ii) the acute and sub chronic effects of mercury on this species were previously investigated in laboratorial conditions providing the basis for this study (Baudrimont *et al*., 1997; Achard *et al*., 2004; Oliveira *et al*., 2015b); and (iii) the species is consumed by humans in several areas (Peng *et al*., 2017). Therefore, the research on the toxicity of mercury to *C. fluminea*, the ability of the species to recover from mercury exposure, and the depuration of the metal in this bivalve is of main interest.

4.2. Material and methods

4.2.1. Chemicals

Mercury chloride (ACS reagent ≥ 99.5% purity, CAS number 7487-94-7) was purchased from Sigma-Aldrich (Germany). The chemicals used in the analyses of biomarkers were purchased from Sigma-Aldrich (Germany). The Bradford reagent was from Bio-Rad Laboratories. (Germany). All the chemicals used in this experiment were of analytical grade.

4.2.2. Collection and maintenance of *C. fluminea* in the laboratory

C. fluminea was collected in the autumn in the estuaries of Minho (M-est) and Lima (L-est) Rivers, located in the NW coast of the Iberian Peninsula (M-est: ~42°30′22.51″N 8°32′ 22.51″W; L-est: 41°42′07.03″N 8°44′37.05″W). These two populations were selected because they have been studied in relation to their contamination by several metals including mercury (Reis *et al*., 2014), to the health status in relation to abiotic variation (Oliveira *et al*., 2015a), population distribution, biomass, impacts on native species and ecosystem functioning, among other aspects (Sousa *et al*., 2006b, 2008e; Ilarri *et al*., 2014; Novais *et al*., 2016). No significant genetic differences between *C. fluminea* populations of M-est and L-est were found so far (Sousa *et al*., 2007; Gomes *et al*., 2016).

The two estuaries have several differences (*e.g*. in the levels and annual variation patterns of several physical and chemical variables, including some nutrients and some environmental contaminants (Gravato *et al*., 2010; Baeta *et al*., 2017), with the L-est being in general more contaminated than the M-est. The M-est is included in NATURA 2000, and is considered globally as a low impacted estuary, despite having some focus of pollution (Sousa *et al*., 2008e; Reis *et al*., 2009; Guimarães *et al*., 2012). Generally, and in relation to the M-est, the L-est is more contaminated due to anthropogenic activities (including a paper mill and a harbour), contributing to higher sediment and water concentrations of several environmental contaminants (Costa-Dias *et al*., 2010; Gravato *et al*., 2010; Guimarães *et al*., 2012). Baeta *et al*. (2017) reported higher levels of δ15N in *Pomatochistus microps* larvae of the L-est than in animals of the same species inhabiting the M-est, indicating a higher input of nitrogen from anthropogenic sources in the L-est. Moreover, this study also reported higher levels of ammonium, nitrate and chlorophyll *a* in the L-est, indicating an overall poorer water quality in the L-est than in the M-est. Such differences provide an opportunity to investigate the influence of long-term population exposure to distinct environmental conditions on the effects and recovery from mercury exposure through the comparison of results from the bioassays carried out with individuals from the two populations. This approach was previously used to evaluate the influence of environmental conditions on the health status of different aquatic species inhabiting these estuaries (Gravato *et al*., 2010; Guimarães *et al*., 2012; Rodrigues *et al*., 2012).

Bivalves were collected using a rake, at low tide, and transported as soon as possible to the laboratory in thermally isolated boxes with water from the collection site. Once in the laboratory, the specimens were put in tanks filled with 16 L of dechlorinated water for human consumption (hereafter indicated as clean medium) in groups of 16 per tank. Animals from both estuaries were maintained separately in these conditions for 14 days (acclimation period) in a room with controlled photoperiod (16 h light: 8 h dark) and temperature (16 ± 2 °C). The clean medium was renewed every 48 h and the animals were fed with *Chlorella vulgaris* and *Chlamydomonas reinhardtii* (50%: 50% cells/cells) in a total concentration of 8×10^5 microalgae cells/mL/bivalve. Temperature, pH, conductivity and dissolved oxygen were monitored at the beginning of the acclimation period and every 24 h with a multi-parametric probe (Multi 340i/Set Wissenschaftlich-Technische Werkstätten, Germany).

4.2.3. Bioassays

The bioassays were carried out under the temperature and photoperiod indicated in section 4.2.2. The experimental design of each bioassay (Fig. 6) included the following treatments: (a) no exposure, *i.e.* animals analysed for biomarkers immediately after the

acclimation period (Ctr0), (b) 8 days of exposure to clean medium (Ctr8), (c) 14 days of exposure to clean medium (Ctr14), (d) 8 days of exposure to 31 µg/L of mercury (Hg8), (e) 14 days of exposure to 31 µg/L of mercury (Hg14) and (f) 8 days of exposure to 31 µg/L of mercury followed by 6 days of exposure to medium without mercury (recovery).

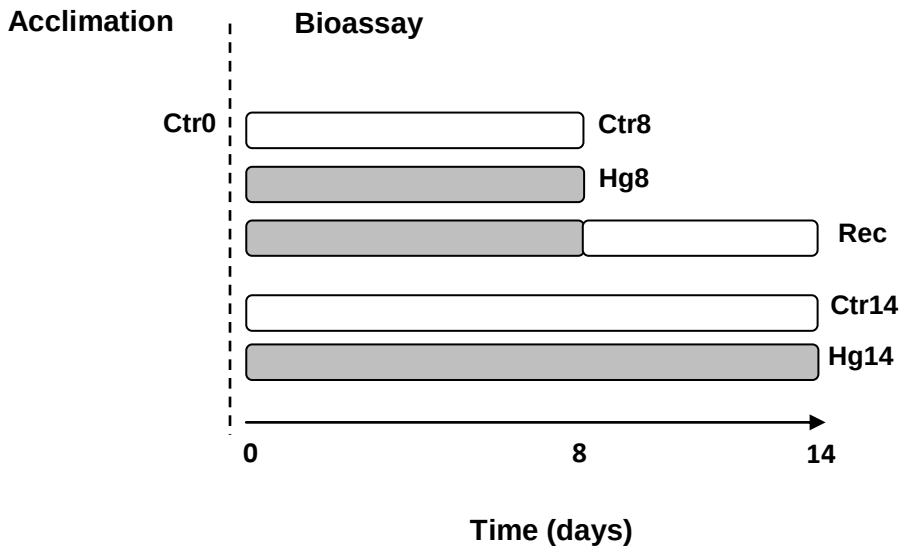

Fig. 6. Experimental design adopted to study the effects of mercury exposure and recovery (rec) in *Corbicula fluminea* from Minho and Lima estuaries. In both bioassays, organisms were analysed after the acclimation period (Ctr0) and after 8 and 14 days of experiment. Bivalves were exposed to the following treatments: 8 days to dechlorinated tap water for human consumption (clean medium) (Ctr8), 14 days to clean medium (Ctr14), 8 days to 31 µg/L of Hg (Hg8), 14 days to 31 µg/L of Hg (Hg14) and 31 µg/L of Hg for 8 days + 6 days to clean medium (Rec).

In each bioassay, 9 bivalves were randomly assigned to each treatment and they were exposed individually in 2 L glass beakers containing 1.8 L of test medium that was renewed at 48 h intervals. Additional air was continuously supplied and animals were feed as indicated for the acclimation period (section 4.2.2). Test medium temperature, pH, conductivity and dissolved oxygen were monitored every 24 h with a multi-parametric probe (section 4.2.2). Just before the starting of the bioassay and after 8 and 14 days of exposure, the biomarkers used as effect criteria (sections 4.2.4 and 4.2.5) were determined according to the experimental design.

4.2.4. Oxygen consumption rate

The OCR of *C. fluminea* was determined individually, immediately after the acclimation period (Ctr0) or after the exposure period (8 or 14 days according to the treatments). The system used consisted in acrylic glass chambers (19 cm × 14 cm × 12 cm). The general procedure followed Rosa *et al*. (2013), with small adaptations and validation to *C. fluminea*, was used. Briefly, each chamber filled with 4 L of clean medium was closed and put in a plastic box with 95 L capacity filled with 70 L of clean medium (the chamber was completely submerged). The circulation of clean medium inside the chambers (flow rate = 200 mL/s) was made through a pump (camper water pump, Eco-plus 12 V, Comet, Florida, USA) powered by a battery (12 V, 60 Ah, 510 A, DiaMec, China) connected to each chamber, allowing a homogeneous distribution of oxygen. One bivalve was carefully put in each chamber. The determinations of dissolved oxygen (DO, mg/L) were made at the beginning of the test (DOi) and after one hour (DOf) with an oxygen probe (Oxi 320/Set Wissenschaftlich-Technische Werkstätten, Germany). The OCR was calculated as:

$$OCR = (DOi) - (DOf) / t \times n$$

DOi and DOf are the dissolved oxygen measured at the end and at the beginning of the test, respectively, *t* is the time of the test (hours) and *n* is the number of organisms per chamber. The OCR was expressed in mg of O_2 consumed/L/hour/ bivalve.

4.2.5. Biochemical biomarkers

After OCR determinations, animals were sacrificed and the following tissues were isolated on ice: adductor muscle for ChE activity, foot for IDH and ODH activities, and gills for the activities of CAT, GR, GPx and GST, and LPO levels. Samples were maintained at −80 °C until further analysis. The preparation of samples and biomarkers determinations were made as described in Oliveira *et al*. (2015b). Briefly, in the day of determination, the tissues were unfrozen on ice and homogenized (Ystral GmbH d-7801, Dottingen, Germany) in cold buffers. One part of the gill homogenate (200 µL) was directly used for the determination of LPO levels, through the quantification of thiobarbituric acid-reactive substances (TBARS) at 535 nm (Ohkawa *et al*., 1979; Bird and Draper, 1984; Filho *et al*., 2001; Torres *et al*., 2002). The remaining homogenates were centrifuged in a 3K30 Laboratory Centrifuge (Sigma, Germany) and the supernatants were collected. The protein concentration of gill supernatant was standardized to 4 mg/mL and the adductor muscle and foot supernatants were standardized to 1 mg/mL. The protein content of the samples was determined according to the Bradford dye-binding method (Bradford, 1976)

at 600 nm, adapted to microplate (Frsco and Guilhermino, 2002), using bovine γ-globulin as protein standard. CAT was determined in gills supernatant according to Clairborne (1985) at 240 nm. GR activity was determined in gills supernatant at 340 nm, according to Carlberg and Mannervik (1985). GPx activity was assessed in gills supernatant according to Mohandas *et al*. (1984) at 340 nm. GST activity was determined gills supernatant at 340 nm using 1-chloro-2,4-dinitrobenzene (CDNB) according to Habig *et al*. (1974) and Frasco and Guilhermino (2002). ChE activity was determined in adductor muscle supernatant at 412 nm according to Ellman *et al*. (1961) and Guilhermino *et al*. (1996). IDH activity was determined in foot supernatant at 340 nm according to Ellis and Goldberg (1971) and Lima *et al*. (2007). ODH activity was determined in gills supernatant at 340 nm as described in Livingstone *et al*. (1990) and Lima *et al*. (2007). At the end of sub-individual biomarkers determinations, the protein content of the samples was verified (as previously indicated). Enzymatic activities were expressed in nanomoles of substrate hydrolysed per minute per mg of protein (nmol/min/mg protein) except CAT activity that was expressed in micromoles of substrate hydrolyzed per minute per mg of protein (µmol/min/mg protein). LPO levels were expressed in nanomoles of TBARS per mg of protein (nmol TBARS/mg protein). All sub-individual biomarkers determinations were carried out at 25 °C in a Spectramax® M2 spectrophotometer (Molecular Devices, U.S.A.).

4.2.6. Statistical analysis

For each biomarker, data from Ctr0 of M-est and L-est were compared using the Student's *t*-test. Other data sets (one per biomarker) were tested for normal distribution departures and homogeneity of variances using the Shapiro-Wilk and Levene's tests, respectively. When the assumptions of the Analysis of Variance (ANOVA) were not achieved, appropriated data transformations were made. Data of each biomarker was analysed by three-way ANOVA with interactions in relation to: (i) the origin of organisms (M-est or L-est) (ii) the time of laboratory exposure (0, 8 or 14 days), which includes organisms not exposed to mercury, those exposed to mercury and recovery and (iii) the type of exposure (to clean medium, to mercury and to the recovery treatment), which includes organisms analysed at 0, 8 and 14 days. The fixed factors are hereafter indicated as: "estuary", "time" and "exposure", respectively. Data from distinct estuaries were further analysed separately. For each estuary and each biomarker, different treatments were compared through a one-way ANOVA followed by the Tukey's multi-comparison test when significant differences were found. The analyses were carried out in SPSS statistics version 23.0 for Windows (IBM®, U.S.A). The significance level was 0.05.

4.3. Results

The temperature and pH variations in each test beaker were always lower than 0.5 °C and 0.1 pH units, respectively, and the test medium DO was always higher than 9.5 mg/L. No mortality was recorded during the bioassays. At the beginning of the bioassays (Ctr0) and for all biomarkers, no significant differences between bivalves from distinct estuaries were found (Table 5).

Table 5. Results of the Student's *t*-test performed to compare the size and biomarkers of *Corbicula fluminea* from the Minho (M-est) and Lima (L-est) River estuaries at the beginning of the bioassays (Ctr0). Values are the mean ± standard error of anterior-posterior shell length (size), oxygen consumption rate (OCR), cholinesterase enzymes (ChE) activity, NADP-dependent isocitrate dehydrogenase (IDH) activity, octopine dehydrogenase (ODH) activity, catalase (CAT) activity, glutathione reductase (GR) activity, glutathione peroxidase (GPx) activity, glutathione *S*-transferases (GST) activity, and lipid peroxidation (LPO) levels.

Parameter	M-est	L-est	Student *t*-test
Size	28.2 ± 0.11	28.6 ± 0.14	t_{16} = -1.93 ; p = 0.06
OCR	0.47 ± 0.02	0.45 ± 0.02	t_{16} = 0.98 ; p = 0.89
ChE	20.5 ± 2.1	21.2 ± 1.6	t_{16} = - 2.78 ; p = 0.73
IDH	3.1 ± 0.22	3.2 ± 0.24	t_{15} = - 0.11 ; p = 0.70
ODH	4.1 ± 0.35	4.8 ± 0.33	t_{16} = -1.40 ; p = 0.86
CAT	3.1 ± 0.37	3.6 ± 0.48	t_{16} = - 0.86 ; p = 0.26
GR	3.2 ± 0.37	3.0 ± 0.46	t_{16} = 0.30 ; p = 0.98
GPx	2.1 ± 0.24	1.9 ± 0.22	t_{15} = 0.71 ; p = 0.64
GST	16.1 ± 1.72	13.6 ± 0.46	t_{15} = 1.22 ; p = 0.21
LPO	0.36 ± 0.06	0.33 ± 0.04	t_{16} = 0.42 ; p = 0.36

The integrated analysis of each biomarker data through a 3-way ANOVA (Table 6) indicated a significant main effect of estuary in OCR and in the activity of the enzymes IDH, CAT, GR and GPx, whereas no significant differences were found for the other biomarkers. A significant main effect of time was found in OCR and LPO levels. The exposure conditions had a significant main effect in OCR, in the activities of IDH, GR and GST, and in LPO levels. A significant interaction between estuary and time was found in GR and GST activities. A significant interaction between estuary and exposure was found in OCR and GR activity. A significant interaction between time and exposure was found in

OCR and LPO levels. A significant interaction among estuary, time and exposure was found in GR and GST activities. No other significant interactions were observed. Due to these significant differences and interactions, data from distinct estuaries were further analysed separately.

Table 6. Results of the three-way ANOVA performed to investigate the effects of estuary (Est), time of exposure (Time) and type of exposure (Exp) on the biomarkers of *Corbicula fluminea*. Oxygen consumption rate (OCR), activities of cholinesterase enzymes (ChE), NADP-dependent isocitrate dehydrogenase (IDH), octopine dehydrogenase (ODH), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), glutathione *S*-transferases (GST), and lipid peroxidation (LPO) levels. Df - Degrees of freedom.

Source of variation		**OCR**	**CHE**	**IDH**	**ODH**	**CAT**	**GR**	**GPx**	**GST**	**LPO**
	df	(1, 106)	(1, 103)	(1, 101)	(1, 105)	(1, 105)	(1, 102)	(1, 106)	(1, 101)	(1, 102)
Estuary	F	3.20	0.51	4.45	0.199	20.95	4.88	19.97	0.18	0.34
	p	0.08	0.48	0.04	0.66	0.00	0.03	0.00	0.67	0.56
	df	(2, 106)	(2, 103)	(2, 101)	(2, 105)	(2, 105)	(2, 102)	(2, 106)	(2, 101)	(2, 102)
Time	F	15.1	1.35	1.02	1.01	1.02	1.19	2.52	1.30	11.6
	p	0.00	0.26	0.36	0.37	0.36	0.15	0.09	0.26	0.00
	df	(2, 106)	(2, 103)	(2, 101)	(2, 105)	(2, 105)	(2, 102)	(2, 106)	(2, 101)	(2, 102)
Exposure	F	14.0	1.04	9.16	0.42	0.92	10.9	0.88	10.1	4.32
	p	0.00	0.36	0.00	0.66	0.40	0.00	0.42	0.00	0.02
	df	(2, 106)	(2, 103)	(2, 101)	(2, 105)	(2, 105)	(2, 102)	(2, 106)	(2, 101)	(2, 102)
Est x Time	F	0.27	0.27	0.81	1.08	1.83	7.31	1.74	10.3	0.95
	p	1.00	0.76	0.45	0.35	0.17	0.001	0.18	0.002	0.39
	df	(2, 106)	(2, 103)	(2, 101)	(2, 105)	(2, 105)	(2, 102)	(2, 106)	(2, 101)	(2, 102)
Est x Exp	F	3.02	0.292	0.81	0.90	0.306	17.79	0.71	0.59	1.76
	p	0.05	0.75	0.45	0.10	0.74	0.00	0.50	0.56	0.18
	df	(1, 106)	(1, 103)	(1, 101)	(1, 105)	(1, 105)	(1, 102)	(1, 106)	(2, 101)	(1, 102)
Time x Exp	F	19.7	0.000	1.30	1.26	0.002	0.22	0.19	0.59	7.28
	p	0.00	1.00	0.26	0.27	0.96	0.64	0.66	0.56	0.01
	df	(1, 106)	(1, 103)	(1, 101)	(1, 105)	(1, 105)	(1, 102)	(1, 106)	(1, 101)	(1, 102)
Est x Time x Exp	F	0.39	0.529	0.010	0.599	0.324	4.70	0.92	5.20	1.62
	p	0.39	0.47	0.92	0.44	0.57	0.03	0.34	0.03	0.70

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4.3.1. Effects of mercury and recovery in *C. fluminea* from the M-est

The results of the biomarkers determined in M-est bivalves are shown in Table 7 and Figs. 7 and 8. Significant differences in the OCR, in the activity of the enzymes ChE, IDH, GR and GST, and in LPO levels among treatments were found, whereas no significant differences in ODH, CAT and GPx activities were observed (Table 7).

Table 7. Results of the one-way ANOVA carried out with the data of each biomarker to compare different experimental treatments. M-est - Minho River estuary; L-est - Lima River estuary; OCR - Oxygen consumption rate; ChE - Cholinesterase enzymes activity; IDH - NADP-dependent isocitrate dehydrogenase activity; ODH - Octopine dehydrogenase activity; CAT - Catalase activity; GR - Glutathione reductase activity; GPx - Glutathione peroxidase activity; GST - Glutathione *S*-transferases activity; LPO - Lipid peroxidation levels; df - Degrees of freedom.

Biomarker	Estuary	df	F	p
OCR	M-est	(5, 54)	10.81	0.000
	L-est	(5, 54)	9.31	0.000
ChE	M-est	(5, 53)	4.22	0.003
	L-est	(5, 52)	0.67	0.997
IDH	M-est	(5, 52)	2.74	0.003
	L-est	(5, 52)	2.82	0.027
ODH	M-est	(5, 53)	0.75	0.588
	L-est	(5, 53)	0.68	0.664
CAT	M-est	(5, 52)	0.75	0.593
	L-est	(5, 54)	1.65	0.164
GR	M-est	(5, 51)	9.00	0.000
	L-est	(5, 53)	2.80	0.027
GPx	M-est	(5, 52)	1.19	0.330
	L-est	(5, 53)	1.98	0.099
GST	M-est	(5, 53)	7.22	0.000
	L-est	(5,52)	3.95	0.005
LPO	M-est	(5, 52)	5.06	0.001
	L-est	(5, 54)	2.26	0.034

In all biomarkers, no significant differences among Ctr0, Ctr8 and Ctr14 were found. In relation to the control groups, after 8 days of exposure to mercury, bivalves had significantly decreased GR activity (45%) (Fig. 8A) and no other significant alterations. In relation to control groups, after 8 days of exposure to mercury and 6 additional days in clean medium, the recovery group had significantly decreased OCR (54%) (Fig. 7A), inhibited IDH (40%) (Fig. 7C) and GR activities (52%) (Fig. 8A), significantly increased LPO levels (23%) (Fig. 8C) and no significant differences in the other biomarkers.

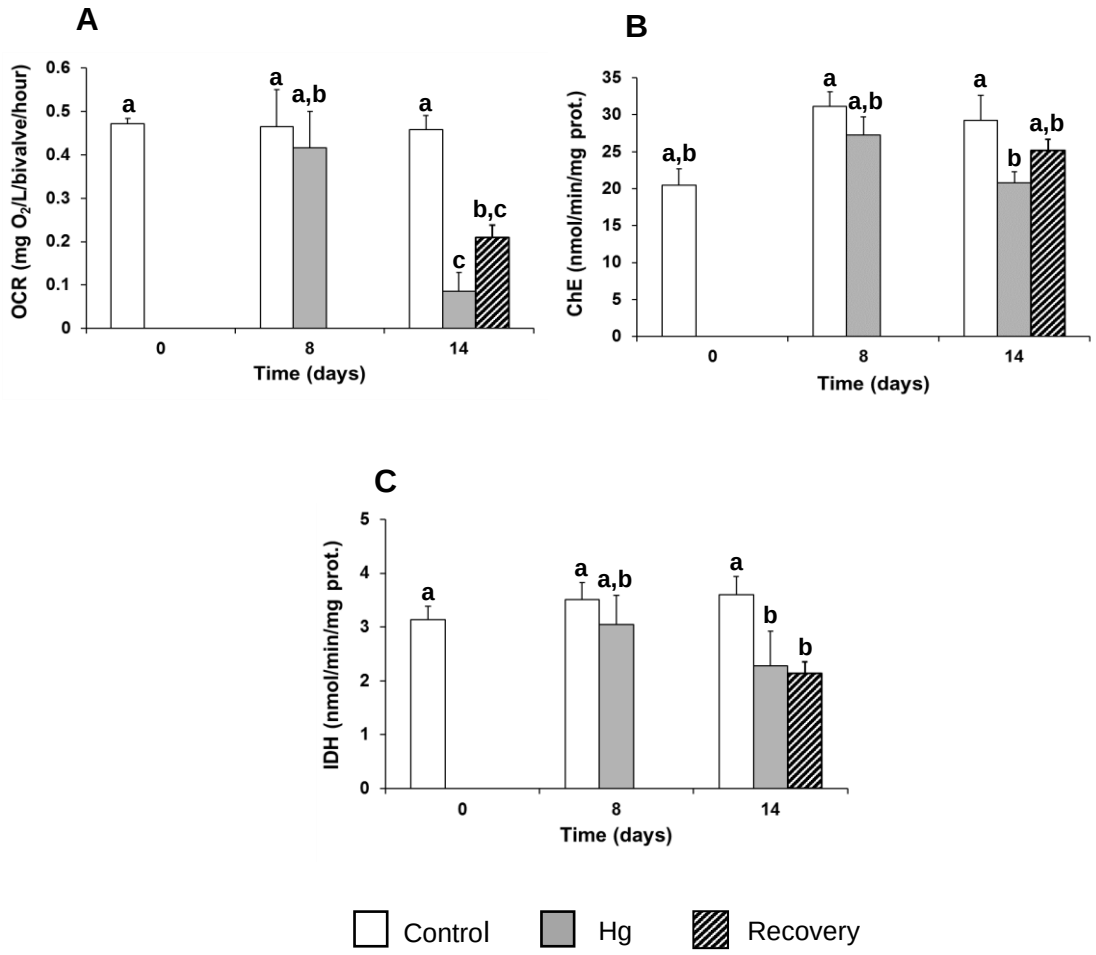

Fig. 7. A - Oxygen consumption rate (OCR), B – Activity of cholinesterase enzymes (ChE) and C – Activity of NADP-dependent isocitrate dehydrogenase (IDH) determined in *Corbicula fluminea* from Minho River estuary at day 0 (Ctr0), after 8 and 14 days of exposure to mercury (Hg) and after a period of recovery. Values are the mean ± standard error of 9 organisms. Significant differences between treatments are identified by different letters above the bars (one-way ANOVA and the Tukey's test, $p \leq 0.05$).

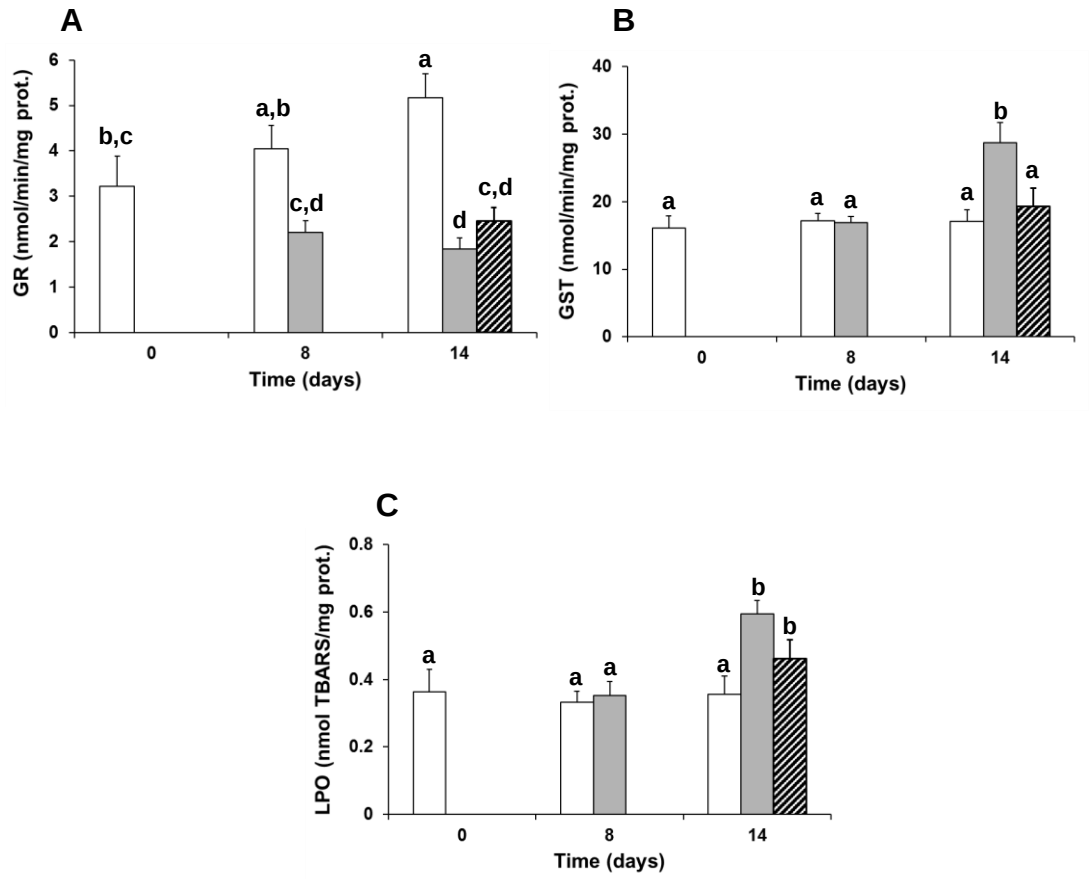

Fig. 8. A - Activity of glutathione reductase (GR), B - Activity of glutathione *S*-transferases (GST) and C - Lipid peroxidation (LPO) levels determined in *Corbicula fluminea* from Minho River estuary at day 0 (Ctr0), after 8 and 14 days of exposure to mercury (Hg) and after a period of recovery. Values are the mean ± standard error of 9 organisms. Significant differences between treatments are identified by different letters above the bars (one-way ANOVA and the Tukey's test, $p \leq 0.05$).

After 14 days of exposure to mercury and in relation to control groups, animals had significantly decreased OCR (81%) (Fig. 7A), IDH (37%), (Fig. 7C) and GR (64%) (Fig. 8A), and increased GST activity (41%) (Fig. 8B) and LPO levels (40%) (Fig. 8C). Animals exposed for 14 days to mercury had lower ChE activity (28%) (Fig. 7B) than those of the Ctr14 treatment, but the effects were not significantly different those of the Ctr0 treatment. No significant differences in any biomarker between animals exposed for 14 days to mercury and those from the recovery treatment were found. Significant differences between animals exposed for 8 days and 14 days were found in OCR, GST activity and LPO levels. During the experiments, animals exposed to treatments containing mercury closed the valves for some periods of time.

4.3.2. Effects of mercury and recovery in *C. fluminea* from the L-est

The results of biomarkers determined in organisms collected from L-est are shown in Table 7 and Fig. 9. No significant differences in any biomarker among the control treatments (Ctr0, Ctr8 and Ctr14) were observed. Significant differences among experimental treatments were found for OCR, IDH, GR and GST activities and LPO levels, whereas the activities of ChE, ODH, CAT and GPx were not significantly different (Table 7).

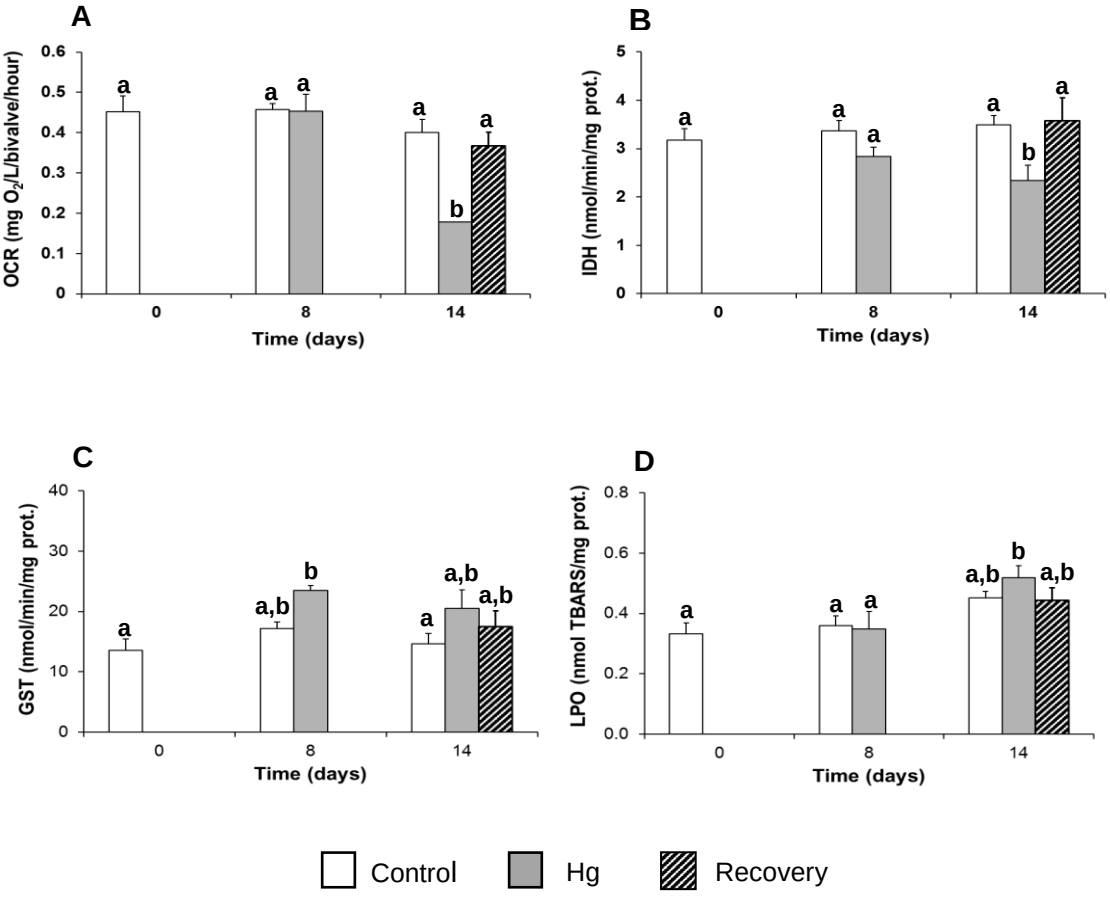

Fig. 9. A - Oxygen consumption rate (OCR), B - Activity of NADP-dependent isocitrate dehydrogenase (IDH), C - Activity of glutathione *S*-transferases (GST) and D - Lipid peroxidation (LPO) levels determined in *Corbicula fluminea* from Lima River estuary at day 0 (Ctr0), after 8 and 14 days of exposure to mercury (Hg) and after a period of recovery. Values are the mean ± standard error of 9 organisms. Significant differences between treatments are identified by different letters above the bars (one-way ANOVA and the Tukey's test, $p \leq 0.05$).

After 8 days of exposure no significant differences were observed for any biomarker. Bivalves exposed to mercury for 14 days, in relation to control groups, had significantly reduced OCR (56%) (Fig. 9A), decreased IDH activity (32%) (Fig. 9B) and increased GST activity (29%) (Fig. 9C). The OCR, IDH activity and LPO levels of bivalves exposed to mercury for 8 days were significantly different from those exposed for 14 days. No significant differences were found in any biomarker determined in organisms of the recovery treatment in comparison to controls. As observed in M-est bivalves, animals exposed to mercury closed the valves for some periods of time.

4.4. Discussion

The lack of significant differences between animals from distinct estuaries at the beginning of the experiments (Ctr0, Table 5) indicates that they had a comparable health *status* at this time. For both estuaries, the lack of significant differences in the biomarkers among animals of Ctr0, Ctr8 and Ctr14 groups indicate that animals were maintained in adequate conditions during the experimental period, and that any differences between these groups and the other ones were due to distinct exposure conditions. Overall, from the results of the 3 way-ANOVA, it can be concluded that animals from distinct estuaries have different sensitivities to mercury and that exposure time and the type of treatment influence the effects on some of the biomarkers.

Regarding the M-est, the reduction of GR activity in bivalves exposed for 8 days to mercury indicates impairment of the activity of this antioxidant enzyme that may cause a reduced capability to respond to oxidative stress. In fact, GR is essential for the maintenance of the ratio between reduced and oxidized forms of glutathione (Jozefczak *et al*., 2012). Thus, its inhibition may compromise the redox cycling and disturb the antioxidant defence system functioning. Moreover, since GR uses NADPH as a source of reduced equivalents, a decrease of its activity likely decreases the production of NADP, which is required for the functioning of other enzymes (Pai and Schulz, 1983; Carugo and Argos, 1997).

The decrease of OCR and IDH activity and the increase of LPO levels in animals of the recovery treatment indicate delayed toxicity induced by 8 days of exposure to the metal that became evident only several days after the end of the exposure. Moreover, because M-est recovery animals had significant differences in OCR, IDH activity and LPO levels in relation to the control groups but not relatively to animals exposed to mercury for 14 days, one can conclude that 6 days in clean medium was not enough to reverse the toxic effects induced by 8 days of exposure to mercury. In L-est animals, no evidences of mercury-induced toxicity

after 8 days of exposure or of delayed toxicity were observed indicating that they were less sensitive to short-term exposure to mercury than those of the M-est.

After 14 days of exposure to mercury, the reduction of the OCR in both M-est and L-est animals indicate a decreased individual fitness. Moreover, decreased OCR and the inhibition of IDH activity without increase of ODH activity suggest hypoxia in animals of both estuaries. The frequent and long-lasting valve closure behaviour observed in animals exposed to mercury, particularly in those exposed for 14 days to the metal, supports this hypothesis. Hypoxia likely results in reduced energy obtained from aerobic pathways of cellular energy production and if not compensate through the activation of anaerobic pathways of energy production, animals will have less energy available. In such conditions, they may need to allocate the energy available to basic functions (*e.g.* basic metabolism, maintenance and repair) compromising functions that are determinant for individual and population fitness such as growth and reproduction (Sokolova *et al*., 2012).

M-est animals exposed for 14 days to mercury also had 28% of ChE inhibition in relation to the Ctr14 group but no significant differences in relation to the Ctr0. In a previous laboratorial study where *C. fluminea* collected in the same site in the M-est was exposed up to 500 µg/L of mercury for 96 h, no significant anticholinesterase effects were found (Oliveira *et al*., 2015b). Thus, despite the well known anticholinesterase effects of mercury in several species (Frasco *et al*., 2005, 2007), the inhibition observed in *C. fluminea* exposed for 14 days to mercury was not considered relevant. In L-est animals, no evidences of neurotoxicity were found.

The significant induction of GST activity, one of the antioxidant enzymes, found in M-est individuals after 14 days of mercury exposure suggests oxidative stress. This hypothesis is supported by the significant increase of LPO levels in these animals indicating lipid oxidative damage. The inhibition of GR activity and of IDH activity may have contributed to the failure of the antioxidant defences in preventing lipid oxidative damage to occur. Thus, in M-est animals, mercury induced oxidative stress and damage, whereas no evidences of such effects in L-est bivalves were found.

The reduction in ORC, inhibition of IDH and induction of oxidative stress and damage by mercury in M-est *C. fluminea* are in good agreement with the findings of previous studies with bivalves. For example, a 50% decrease in OCR was previously found in *Perna viridis* exposed to 0.059 ppm of mercury for 96 h (Mohan *et al*., 1986). IDH inhibition was previously found in *C. fluminea* exposed for 96 h to 0.5 mg/L of mercury (Oliveira *et al*., 2015b). Mercury-induced oxidative stress and damage were previously found in *C. fluminea* (Oliveira

et al., 2015b) and in in other bivalves such as *Scrobicularia plana* and *P. viridis* (Verlecar *et al*., 2008; Ahmad *et al*., 2011).

The results of the present study indicate that bivalves from the M-est were more sensitive to mercury exposure than those from the L-est. Because the acclimation and experimental conditions were similar for animals of the two estuaries and they had a comparable heath condition at the begging of the bioassays, the difference of sensitivity to mercury found may have been due to long-term exposure of the two populations to distinct environmental conditions in their original habitats. In fact, the two estuaries have several differences, including in the concentrations of several environmental contaminants, including metals (Gravato *et al*., 2010; Guimarães *et al*., 2012; Rodrigues *et al*., 2014). Therefore, the L-est population may have developed mechanisms of resistance against metal stress resulting in a decreased sensitivity to mercury. The more likely ones are mechanisms decreasing mercury uptake, increased elimination of the metal, and decrease of the susceptibility of molecular targets. For instance, induction of a multixenobiotic resistance mechanism was previously found in *C. fluminea* after exposure to heavy metals including mercury (Achard *et al*., 2004). Moreover, because the two estuaries also have differences in other parameters of ecological importance, including in the availability of food and nutrients adequate for *C. fluminea* (Oliveira *et al*., 2015a; Baeta *et al*., 2017), such parameters may have also contributed to the differences of sensitivity to mercury between M-est and L-est animals.

4.5. Conclusions

Exposure of *C. fluminea* from the M-est to 31 µg/L of mercury for 8 days caused inhibition of GR activity and delayed toxicity but no toxic effects were observed in bivalves from the L-est. Recovery for 6 days in clean medium was not enough to revert the toxic effects caused by 8 days of exposure to mercury in M-est bivalves. After 14 days of exposure to mercury, animals from both estuaries had decreased OCR and IDH activity, suggesting hypoxia and decrease of the energy obtained through aerobic pathways. Therefore, exposure to 31 µg/L of mercury for 14 days decreased the individual fitness of *C. fluminea* from both estuaries. Moreover, M-est animals had increased GST activity and LPO levels, indicating oxidative stress and damage. Such effects were not observed in L-est bivalves. Thus, M-est organisms were more sensitive to mercury than those of the L-est. The differences of sensitivity between M-est and L-est animals were likely due, at least in part, to long-term exposure of the two populations to distinct environmental conditions.

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CHAPTER V

Effects of microplastics and mercury in the freshwater bivalve *Corbicula fluminea* (Müller, 1774): filtration rate, biochemical biomarkers and mercury bioconcentration

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Abstract

The main objectives of this study were to investigate the effects of a mixture of microplastics and mercury on *Corbicula fluminea,* the post-exposure recovery, and the potential of microplastics to influence the bioconcentration of mercury by this species. Bivalves were collected in the field and acclimated to laboratory conditions for 14 days. Then, a 14-day bioassay was carried out. Bivalves were exposed for 8 days to clean medium (control), microplastics (0.13 mg/L), mercury (0.03 mg/L) and to a mixture (same concentrations) of both substances. The post-exposure recovery was investigated through 6 additional days in clean medium. After 8 and 14 days, the following endpoints were analysed: the post-exposure filtration rate (FR); the activity of cholinesterase enzymes (ChE), NADP-dependent isocitrate dehydrogenase (IDH), octopine dehydrogenase, catalase, glutathione reductase, glutathione peroxidase and glutathione *S*-transferases (GST), and the levels of lipid peroxidation (LPO). After 8 days of exposure to mercury, the bioconcentration factors (BCF) were 55 in bivalves exposed to the metal alone and 25 in bivalves exposed to the mixture. Thus, microplastics reduced the bioconcentration of mercury by *C. fluminea*. Bivalves exposed to microplastics, mercury or to the mixture had significantly ($p \leq 0.05$) decreased FR and increased LPO levels, indicating fitness reduction and lipid oxidative damage. In addition, bivalves exposed to microplastics alone had significant ($p \leq 0.05$) reduction of adductor muscle ChE activity, indicating neurotoxicity. Moreover, bivalves exposed to mercury alone had significantly ($p \leq 0.05$) inhibited IDH activity, suggesting alterations in the pathways of cellular energy production. Antagonism between microplastics and mercury in FR, ChE activity, GST activity and LPO levels was found. Six days of post-exposure recovery in clean media was not enough to totally reverse the toxic effects induced by the substances or to eliminate completely the mercury from the bivalves' body. These findings have implications to animal, ecosystem and human health.

Keywords: Microplastics, Mercury, *Corbicula fluminea*, Filtration rate, Biochemical biomarkers

5.1. Introduction

Microplastics and mercury are considered global pollutants of high concern regarding their adverse effects on environmental and human health (Wright and Kelly, 2017; Barboza *et al*., 2018a; Guilhermino *et al*., 2018). The concentrations of microplastics that have been reported in natural waters vary considerably (Horton *et al*., 2017), with mean concentrations up to 5.51 ± 9.09 mg/L found in polluted freshwater systems (Lasee *et al*., 2017). Regarding mercury, concentrations in the low ppm range have been found in water, sediment and organisms of polluted sites (Driscoll *et al.*, 2007). Mercury is accumulated in freshwater species and causes a wide range of adverse effects, including neurotoxicity, immunotoxicity, oxidative stress and damage, behaviour alterations, decrease of the filtration rate, growth inhibition, and reproduction impairment (Driscoll *et al*., 2007).

Freshwater animals, including species of human consumption, uptake microplastics from water and accumulate them (Santilo *et al*., 2017). Adverse effects resulting from microplastics exposure have been observed in several freshwater animals, such as *Daphnia magna* (Martins and Guilhermino, 2018; Pacheco *et al*., 2018), *Danio rerio* (Lei *et al*., 2018; Lu *et al*., 2018) and *C. fluminea* (Rochman *et al*., 2017; Guilhermino *et al*., 2018). Moreover, microplastics can be transferred along the food webs from lower to higher trophic levels (Farrel and Nelson, 2013) potentially affecting the human health due the consumption of contaminated species (Santillo *et al*., 2017). Another problem is that ingested microplastics may contain other environmental contaminants (Turner and Holmes, 2015). Additionally, under simultaneous exposure to microplastics and other environmental contaminants, toxicological interactions may occur and modify the type and/or the magnitude of the toxic effects (Chen *et al*., 2017; Guilhermino *et al*., 2018; Pacheco *et al*., 2018; Rainieri *et al*., 2018). The number of studies on the biological effects induced by mixtures of microplastics and other environmental contaminants is still limited, especially in freshwater bivalve species (*e.g*. Rochman *et al*., 2017; Guilhermino *et al*., 2018), and more research is needed.

The main objectives of the present study were to investigate the effects of a mixture of microplastics and mercury on *Corbicula fluminea,* the post-exposure recovery, and the potential of microplastics to influence the bioconcentration of mercury by this species.

5.2. Material and methods

5.2.1. Chemicals

Microplastics and mercury were selected as test substances mainly because they are ubiquitous pollutants of high concern regarding animal, environmental and human health and are common contaminants in a high number of freshwater ecosystems (Driscoll *et al*., 2007; Wright and Kelly, 2017; Rainieri *et al*., 2018). The combined effects of mercury and microplastics were previously investigated in marine fish (Barboza *et al*., 2018b) but to the best of our knowledge they were not investigated in freshwater animals so far.

Mercury chloride (≥ 99.5% purity) was purchased from Sigma-Aldrich (Germany). Microplastics consisted of fluorescent polymer microspheres (lot number: 4-1006-1053), of unknown composition, purchased from Cospheric - Innovations in Microtechnology (U.S.A.). According to manufacturer indications, particles had a diameter between 1 and 5 µm, a density of 1.3 g/cc, red colour, wavelengths of excitation and emission of 575 and 607 nm, respectively, and 1 mg of the product contains ~1.836×10^8 spheres (estimate based on an average diameter of 2 µm). The basic characterization of this type of microplastics was done in a previous study (Pacheco *et al*., 2018). All the other chemicals used were of the highest analytical grade available and purchased from Sigma - Aldrich (Germany), Merck (Germany) and Bio-Rad Laboratories (Germany).

5.2.2. Sampling of *C. fluminea* and acclimation to laboratory conditions

C. fluminea was selected as test organism mainly because its natural populations have been found to be contaminated by microplastics (Su *et al*., 2018), the effects of mercury and microplastics on the species were studied before providing baseline knowledge (Oliveira *et al*., 2015b; Rochman *et al*., 2017; Guilhermino *et al*., 2018), and the species is used for human consumption (Su *et al*., 2018). Furthermore, *C. fluminea* is an exotic invasive species in Europe and several other regions of the world that causes important negative ecological impacts in colonized ecosystems (Sousa *et al*, 2008a). Therefore its use in environmental studies generally has no conservational negative impacts (Guilhermino *et al*., 2018). Moreover, its use in such studies may help to control its bioinvasions and provide crucial information regarding environmental quality helping in the protection of its native competitors.

Adult specimens of *C. fluminea* were collected in the Minho River upper estuary (NW Iberian Peninsula) (~42º03'22.51''N 8º32'22.51''W), at low tide, and transported to the laboratory as previously described in Oliveira *et al*. (2015b).

In the laboratory, the bivalves were measured and weighed. A group of 123 bivalves with anterior-posterior shell length between 27–30 mm was selected for the study. The whole soft body (hereafter indicated as body) of 3 bivalves was removed with a plastic scalpel, dried in absorbent paper, weighed, and the samples were stored at −20 ºC for determination of mercury concentrations (section 5.2.5). The remaining bivalves were acclimated for 14 days in a room with temperature set to 16 ± 1 ºC and photoperiod 16 h light (L): 8 h dark (D). Bivalves were maintained in boxes with 16 bivalves each, containing 16 L of dechlorinated tap water for human consumption (hereafter indicated as clean medium) with continuous aeration provided by air bubbling diffusers. The temperature of clean medium was 16 ± 0.5 ºC. Bivalves were fed daily with a mixture of *Chlorella vulgaris* and *Chlamydomonas reinhardtii* cells (50%: 50% cells/cells) in a total concentration of 8×10^5 cells/day/bivalve, as previously reported (Oliveira *et al*., 2015b). The clean medium was renewed at each 48 h. Clean medium temperature, pH, conductivity, and dissolved oxygen were checked daily (Multi 340i/Set and Oxi 320/Set Wissenschaftlich-Technische Werkstätten probes, Germany).

After the acclimation period, 3 bivalves were prepared for mercury body burden determination, as previously indicated, and the samples were stored at −20 ºC until further analyses (section 5.2.5). The remaining animals were used in the bioassay as described in section 5.2.3.

5.2.3. Experimental design and exposure conditions of the bioassay

The room temperature, test medium temperature and food provided during the bioassay were similar to those of the acclimation period (section 5.2.2). Eight treatments were tested: 8-day control (clean medium only); 8-day exposure to 0.13 mg/L of microplastics (8-day microplastics); 8-day exposure to 0.03 mg/L of mercury (8-day mercury); 8-day exposure to a mixture of microplastics (0.13 mg/L) and mercury (0.03 mg/L), hereafter indicated as mixture (8-day mixture); 14-d control (clean medium only); 8-day exposure to 0.13 mg/L of microplastics + 6-day exposure to clean medium (microplastics-recovery); 8-day exposure to 0.03 mg/L of mercury + 6-day exposure to clean medium (mercury-recovery); and 8-day exposure to the mixture + 6-day exposure to clean medium (mixture-recovery). The concentrations of mercury and microplastics indicated are the estimated exposure concentrations, calculated from the actual concentrations of the substances measured in fresh and old media along the bioassay as indicated in section 5.2.5. The concentrations of microplastics and mercury tested were selected based on previous studies (Guilhermino *et al*., 2018; Oliveira *et al*., 2018) and because they are in the range of concentrations reported

for natural waters (Horton *et al*., 2017; Lasee *et al*., 2017). The treatments containing microplastics were prepared daily by dilution of a stock colloidal solution (prepared daily in ultra-pure water) into clean medium. The treatments containing mercury were prepared daily by dilution of a stock solution (prepared daily in ultra-pure water) into clean medium.

Bivalves were exposed individually (*i.e.* 1 bivalve per beaker) in 2 L glass beakers filled with 1.8 L of test media prepared according the treatments, with 13 bivalves per treatment: 9 for biomarkers and 3 for determination of mercury body concentrations. Nine additional bivalves were exposed individually to 0.13 mg/L of microplastics for 8 days in the same conditions to investigate the presence of microplastics in gills and in the digestive system. Moreover, 3 additional beakers without bivalves per treatment were maintained in the same conditions to investigate potential changes in microplastics and mercury concentrations in test medium not due to bivalves. Continuous additional air supply was provided to the beakers helping to maintain microplastics in the water column and all the beakers were covered to prevent mercury and test medium evaporation. Animals were fed daily as indicated in section 5.2.2. Test medium was renewed each 24 h. The abiotic parameters were checked daily, and bivalves were observed at least three times a day.

At the beginning and at the end of the bioassay, and at the time of test medium renewal, samples of test medium were collected for determination of actual (determined) concentrations of microplastics and mercury. Samples were collected immediately after test medium preparation, hereafter indicated as fresh test medium (0 h) and in test medium that remained in the beakers for 24 h, hereafter indicated as old test medium (24 h). The determinations of microplastics were done immediately after sample collection as indicated in section 5.2.5. Samples for mercury determinations were stored at −20 ºC until further analysis.

At the end of the exposure period (8 or 14 days), 24 bivalves (3 bivalves of each treatment, 1 per sample, thus 3 independent replicates per treatment) were prepared for body burden mercury analyses and stored at −20 ºC. From the bivalves exposed to microplastics, 9 were observed for microplastics localization in gills (confocal fluorescence microscopy, DM6000B Leica, Germany) and in the digestive system using a stereoscope (213628 Nikon, Japan). The other bivalves were used for endpoints determination (section 5.2.4).

5.2.4. Endpoints

The parameters chosen to evaluate the effects of microplastics and mercury were the post-exposure filtration rate (FR), lipid peroxidation (LPO) levels and the activity of the enzymes

cholinesterases (ChE), NADP-dependent isocitrate dehydrogenase (IDH), octopine dehydrogenase (ODH), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione *S*-transferases (GST). FR was selected because it indicates the ability of the bivalves to intake food, namely microalgae and other food items from the water column, a crucial function for individual fitness. ChE activity was determined in the adductor muscle. It was used because neuromuscular function is crucial for shell opening and closing, a good cholinergic neurotransmission is fundamental for several other physiological and behavioural functions, and microplastics were found to inhibit *C. fluminea* ChE activity (Guilhermino *et al*., 2018). IDH and ODH were determined in foot tissue and were used because they are involved in the cellular pathways of energy production. CAT, GR GPx and GST activities were determined in gills. They were used as indicative of oxidative stress. LPO levels were determined in gills and were used as indicative of lipid peroxidation damage. Antioxidant enzymes and LPO levels were selected as biomarkers because microplastics and mercury were found to induce oxidative stress and damage in bivalves including in *C. fluminea* (Oliveira *et al*., 2015b; Ribeiro *et al*., 2017).

The FR was determined individually in each animal immediately after the exposure period, based on the removal of microalgae cells from clean medium, according to Coughlan (1969). Briefly, glass beakers filled with 250 mL of fresh clean medium containing *C. vulgaris* (algal suspension) (2.2×10^6 cells/mL) were previously prepared. One bivalve was put into each beaker. The optical density (OD) was measured in triplicate in samples of the algal suspension from each beaker, at 440 nm in a Spectramax® M2 spectrophotometer (Molecular Devices, U.S.A.) to determine the microalgae cell concentration, using a calibration curve (OD *versus* cells concentrations). The time was recorded. After 1 hour, samples of the algal suspension were collected from each beaker and their OD was read in triplicate at 440 nm. The FR was calculated as:

$$FR = [(V/nt) \times \ln (Ci/Cf)]$$

V is the volume of algal suspension prepared in clean medium (mL), *n* is the number of bivalves, *t* is the time (hours), and *Ci* and *Cf* are the concentrations of microalgae (number of cells/mL) at the beginning and after 1 hour, respectively. The FR was expressed in mL of algal suspension/h/bivalve.

After FR determinations, the bivalves were removed from the beakers and left to rest for ~ 2 hours. Then, from each bivalve, tissues were isolated on ice. The adductor muscle and gills

were put in potassium phosphate buffer (0.1M, pH = 7.2) and in potassium phosphate buffer (0.1M, pH = 7.4), respectively. One portion of the foot was put in Tris buffer 50 mM and the other portion was put in Tris buffer 50 mM with ethylenediaminetetraacetate acid disodium salt dehydrate 1 mM and DL-1,4-Dithiothreitol 1 mM. All the samples were stored at – 80 ºC until further analyses. In the day of biochemical biomarkers analyses, samples for these determinations were defrosted on ice and prepared as indicated in Oliveira *et al*. (2015b). The concentration of protein in the supernatants of tissue homogenates was adjusted before the determinations (1 mg/mL for ChE, IDH and ODH activity determinations and 4 mg/mL for antioxidant enzymes and LPO levels). The concentration of protein was determined according to Bradford (1976), adapted to microplate (Frasco and Guilhermino, 2002), using *γ*-globulin as standard protein.

ChE activity was determined in the adductor muscle supernatant at 412 nm according to Ellman *et al*. (1961) and Guilhermino *et al*. (1996). IDH activity was determined in foot supernatant at 340 nm, according to Ellis and Goldberg (1971) with adaptations (Lima *et al*. 2007). ODH activity was determined in foot supernatant at 340 nm as indicated in Livingstone *et al*. (1990) with adaptations (Lima *et al.,* 2007). CAT activity was determined at 240 nm as described in Clairborne (1985). GR activity was determined at 340 nm according to Carlberg and Mannervik (1985). GPx activity was determined at 340 according to Mohandas *et al.* (1984). GST activity was assessed at 340 nm according to Habig *et al.* (1974) with adaptations (Frasco and Guilhermino 2002). LPO levels were assessed through the quantification of thiobarbituric acid reactive substances (TBARS) at 535 nm according to Ohkawa *et al*. (1979) and Bird and Draper (1984) with adaptations (Filho *et al*. 2001; Torres *et al*. 2002).

After biomarkers determinations, the concentration of protein was again determined and the values obtained were used to express the enzymatic activities and LPO levels. The enzymatic activities were expressed in nanomoles of substrate hydrolysed per minute per mg of protein (nmol/min/mg protein), with exception of CAT that was expressed in micromoles of substrate hydrolysed per minute per mg of protein (µmol/min/mg protein). LPO levels were expressed in nanomoles of TBARS per mg of protein (nmol TBARS/mg protein). All the analyses were performed in triplicate, at 25 ºC in a Spectramax® M2 spectrophotometer (Molecular Devices, U.S.A.).

5.2.5. Microplastics and mercury in test media and mercury in *C. fluminea*

The actual concentrations of microplastics in fresh and old media were determined immediately after sample collection, as described in Luís *et al*. (2015) with minor adaptations (Guilhermino *et al*., 2018). Briefly, sample fluorescence was read (575 nm excitation, 607 nm emission) in a spectrofluorimeter Spectramax® M2 (Molecular Devices, U.S.A.). The microplastics concentrations were determined from the fluorescence (FLU) values (F units) using the following linear regression model fitted to a previously determined calibration curve (N = 24, Pearson's correlation coefficient = 0.999, p = 0.000 (Fig. 12, Supplementary material) :

$$\text{Microplastics concentration (mg/L)} = -0.02 + 0.01 \times (\text{F units}),\ R^2 = 99.7\%$$

The decrease of microplastics concentrations in test media along 24 h (time of test media renewal), hereafter indicated as microplastics decay, was calculated directly from the fluorescence readings as follows (Guilhermino *et al*., 2018):

$$\text{Decay (\%)} = 100 - (\text{FLU of fresh test medium} \times 100 / \text{FLU of old test medium})$$

Because more than 20% of microplastics decay during the interval of test medium renewal was found (Table 8), the estimated exposure concentrations of microplastics were calculated from the geometric means of the actual concentrations of fresh and old media collected from the individual beakers at each time of test medium renewal along the bioassay (OECD, 2011).

Mercury was determined in samples of fresh and old test media collected each 24 h and in the body of *C. fluminea.* The detailed procedure is described in Barboza *et al*. (2018b). Briefly, after preparation, samples were analysed by Atomic Absorption Spectrometry according to Costley *et al.* (2000), using a silicon UV diode detector in an automatic Mercury Analyzer (AMA-254, LECO, Czech Republic). The precision error (relative standard deviation of three replicates) was less than 5%. The accuracy of the analytical method was monitored by periodic analyses of a certified standard reference material BCR 463 (mercury and methylmercury in tuna fish). The recovery percent is indicated in Table 13 (Supplementary material). The mercury concentrations in test media and in the body of *C. fluminea* were expressed in mg/L and µg/g wet weight (ww), respectively. Because more than 20% of

mercury decay (*i.e*. decrease of mercury concentrations in test medium) during the interval of test medium renewal occurred (Table 9), the estimated exposure concentrations were calculated as previously indicated for microplastics. For the calculation of the mercury bioconcentration factors (BCF), first the mean mercury concentrations determined in the bivalves of the respective control groups was subtracted from the mean of mercury concentrations determined in bivalves exposed to each of the other treatments containing the metal. Then, each BCF was calculated as:

BCF = mean of body mercury concentrations (ppm) / mean of mercury estimated exposure concentrations in test medium (ppm)

5.2.6. Statistical analysis

The results are indicated as the mean ± standard deviation (SD) or as the mean ± standard error of the mean (S.E.M.). Each data set was checked for normality of distribution and homogeneity of variances using the Shapiro-Wilks and the Levene tests, respectively. Whenever necessary, appropriate transformations were applied. For each data set, different treatments were compared with one-way Analysis of Variance (one way-ANOVA), two-way ANOVA (2 way-ANOVA) or three-way ANOVA (3 way-ANOVA), followed by the multi-comparison Tukey's test. Other comparisons were made using the Student's *t*-test. The significant level was set at 0.05. All statistical analyses were performed using the software IBM SPSS Statistics version 24.0 for Windows (IBM®, U.S.A.).

5.3. Results and discussion

No mortality was recorded during the bioassay. The test medium temperature and pH variation in individual beakers were always lower than 1 ºC and 0.5 pH units, respectively, and the dissolved oxygen in test media was always higher than 8.4 mg/L. Thus, the abiotic conditions were adequate for the maintenance of the bivalves.

5.3.1. Microplastics and mercury in test media

The concentrations of microplastics determined in fresh (0 h) and old (24 h) test medium and the complete results of the statistical analyses are indicated in Table 8. In fresh (0 h) test

medium of beakers without bivalves, the mean (± SD) of microplastics concentration was 0.17 ± 0.01 mg/L in beakers containing the particles alone and 0.17 ± 0.01 mg/L in beakers with the mixture, and no significant differences between them were found (Table 8).

The corresponding values in fresh test medium of beakers with bivalves were 0.18 ± 0.02 mg/L and 0.18 ± 0.02 mg/L, respectively, and no significant differences between them were found (Table 8). These results indicated that mercury did not influence fluorescence readings in freshwater, in good agreement with previous findings (Guilhermino *et al*., 2018).

In old test medium (24 h), the concentrations of microplastics in beakers without bivalves were 0.14 ± 0.01 mg/L in the treatments with the particles alone and 0.12 ± 0.02 mg/L in the mixtures. The corresponding means in old test medium of treatments with bivalves were 0.07 ± 0.02 mg/L and 0.09 ± 0.02 mg/L respectively. The analysis of old test medium data by 2-way ANOVA (Table 8) indicated significant differences between treatments with and without bivalves, no significant differences between treatments with and without mercury, and a significant interaction between the two factors.

The comparison of the microplastics concentrations in fresh (0 h) and old (24 h) test media of treatments containing microplastics alone indicated a higher decay of microplastics in the presence of bivalves (54 %) than in their absence (15%), and a similar finding was observed in the mixtures (50 % and 25%, respectively, Table 8). Overall, these findings indicate uptake of microplastics by *C. fluminea* in agreement with a previous study where the same type of particles and the same species were tested (Guilhermino *et al*., 2018). Moreover, the results suggest that microplastics and mercury interacted in test medium.

Table 8. Actual concentrations of microplastics (MP, mg/L) obtained from fluorescence (relative fluorescence units - RFU) determined in fresh (0 h) and old (24 h) media, in the absence or presence of mercury (Hg) and in the absence or presence of *Corbicula fluminea*. The values are the mean ± standard deviation. A two-way ANOVA was performed to investigate the effect of Hg and animals in MP concentrations. The MP estimated exposure concentrations in test media with or without Hg were compared by the Student's *t*-test. The significant level was 0.05.

Fresh media					
Treatment	Animals	N	Fluorescence	MP concentration	Student's *t*-test
8-day MP	No	24	17.6 ± 1.0	0.17 ± 0.01	t_{46} = 1.016; p = 0.875
8-day Mixture	No	24	17.3 ± 1.0	0.17 ± 0.01	
Overall	-	48	17.4 ± 1.0	0.17 ± 0.01	
8-day MP	Yes	72	18.5 ± 1.6	0.18 ± 0.02	t_{142} = 0.710; p = 0.214
8-day Mixture	Yes	72	18.6 ± 2.2	0.18 ± 0.02	
Overal	-	144	18.5 ± 0.2	0.18 ± 0.02	
Old media					
Hg presence	Animals	N	Fluorescence	MP concentration	Decay (%)
No	No	24	14.9 ± 0.91	0.14 ± 0.01	15
	Yes	72	8.43 ± 2.02	0.07 ± 0.02	54
Yes	No	24	13.0 ± 2.20	0.12 ± 0.02	25
	Yes	72	9.20 ± 1.75	0.09 ± 0.02	50
Factor	Animals	N	Fluorescence	MP concentration	2-way ANOVA
Hg	No	96	10.0 ± 3.33	0.09 ± 0.04	$F_{(1, 192)}$ = 3.41; p = 0.066
	Yes	96	10.1 ± 2.47	0.10 ± 0.03	
Animals	No	48	13.9 ± 1.89	0.13 ± 0.02	$F_{(1, 192)}$ = 2.79; p = 0.000
	Yes	144	8.82 ± 1.92	0.08 ± 0.02	
Interaction					$F_{(1, 192)}$= 19.0; p = 0.000
MP Estimated exposure concentration					
Treatment		N	Fluorescence	MP concentration	Student's *t*-test
8-day MP		144	13.5 ± 5.40	0.11 ± 0.06	t_{286} = -0.704; p = 0.156
8-day Mixture		144	13.9 ± 5.11	0.13 ± 0.05	
Overall		288	13.7 ± 5.20	0.13 ± 0.06	

The decay of microplastics in test medium of beakers without bivalves may have been due to deposition of the particles in the bottom of the beakers because the microplastics tested had a higher density than clean medium, as suggested previously for other microplastics (Cole *et al*., 2011). Microplastics aggregation and sedimentation may have occurred too, contributing to decrease the concentrations of microplastics in test medium. In a smaller magnitude, other processes (*e.g.* adsorption of the particles to the internal surface of glass walls of the beakers) may have also contributed to the microplastics decay found (Luís *et al*., 2015; Barboza *et al*., 2018b). However, the higher decay of microplastics in beakers with bivalves than in beakers with test medium only, indicates uptake of microplastics by the bivalves. The higher decay of microplastics in beakers with the mixture (25%) than in treatments with microplastics alone (15%) in the absence of animals, and the significant interaction between mercury and microplastics in the 2-way ANOVA (Table 8), suggests adsorption of the metal to microplastics, slightly increasing their weight and sedimentation leading to decreased concentrations in test medium.

The mean (± SD) of mercury actual concentrations in fresh (0 h) test medium with and without microplastics were 0.045 ± 0.006 mg/L and 0.042 ± 0.004 mg/L, respectively, and no significant differences between them were found (Table 9). These findings indicate that microplastics did not interfere with the processes of sample preparation and mercury determinations, at least immediately after test medium preparation.

The mean (± SD) of mercury concentrations in old test medium (24 h) of beakers without bivalves and without microplastics was 0.032 ± 0.0002 mg/L, and the mercury decay over 24 h was 24%. This decay may have been due to small losses by evaporation despite the beakers being covered and adsorption to the internal surface of glass walls of the beakers, as suggested in previous studies with this metal (Inza *et al*. 1998). In old test medium (24 h) of beakers without bivalves but with microplastics, the mean (± SD) of mercury concentration was 0.018 ± 0.0003 mg/L. The means (± SD) of mercury concentrations in old test medium of beakers with bivalves were 0.016 ± 0.0012 mg/L and 0.011 ± 0.0009 mg/L in the absence and in the presence of microplastics, respectively. In old test medium, significant differences in the concentrations of mercury between test medium with and without microplastics, between beakers with and without bivalves, and a significant interaction between the two factors were found (2-way ANOVA, Table 9).

Table 9. Actual concentrations of mercury (Hg, mg/L) in fresh (0 h) and old media (24 h) in the absence or presence of microplastics (MP) and in the absence or presence of *Corbicula fluminea.* Values are the mean ± standard deviation. Hg concentrations in fresh media with and without MP were compared by Student's *t*-test. A two-way ANOVA was performed to investigate the effect of MP and animals in Hg concentrations in old media. The Hg estimated exposure concentrations in test media with or without MP were compared by the Student's *t*-test. The significant level was 0.05.

Fresh media				
Treatment	Animals	N	Hg concentration	Student's *t*-test
8-day Hg	No	3	0.042 ± 0.004	t_4 = - 0.91; p = 0.396
8-day Mixture	No	3	0.045 ± 0.006	
Overall	-	6	0.044 ± 0.005	
Old media				
MP presence	Animals	N	Hg concentration	Decay (%)
No	No	3	0.032 ± 0.0002	24
	Yes	3	0.016 ± 0.0012	66
Yes	No	3	0.018 ± 0.0003	61
	Yes	3	0.011 ± 0.0009	74
Factor	Animals	N	Hg concentration	2-way ANOVA
MP	No	6	0.027 ± 0.008	$F_{(1, 11)}$ = 873; p = 0.000
	Yes	6	0.015 ± 0.003	
Animals	No	6	0.025 ± 0.008	$F_{(1, 11)}$ = 1131; p = 0.000
	Yes	6	0.013 ± 0.002	
Interaction				$F_{(1, 11)}$ = 213; p = 0.000
Estimated exposure concentrations				
Treatment		N	Hg concentration	Student's *t*-test
8-day Hg		9	0.031 ± 0.014	t_{16} = 0.767; p = 0.122
8-day Mixture		9	0.025 ± 0.018	
Overall		18	0.028 ± 0.016	

The mercury decay was higher in the presence of bivalves (66% - 74%) than in their absence (24% - 61%) and in the presence of microplastics (61% - 74%) than in their absence (24% - 66%). Overall these findings indicate uptake of mercury by *C. fluminea*, and suggest adsorption of mercury to microplastics, in good agreement with the microplastics findings previously discussed, and with a previous study where decrease of the aqueous percentage of mercury in the presence of another type of microplastics was found (Turner and Holmes, 2015).

The mean of the estimated exposure concentrations were 0.13 ± 0.06 mg/L for microplastics and 0.03 ± 0.02 mg/L for mercury. Such concentrations are ecological relevant because mean concentrations of microplastics up to 5.51 ± 9.09 mg/L (Lasee *et al*., 2017) and of mercury up to the low ppm range (Driscoll *et al*., 2007) were found in environmental freshwaters.

5.3.2. Microplastics and mercury in the body of *C. fluminea*

Microplastics were detected along the digestive tract and in the gills of *C. fluminea* exposed for 8 days to microplastics (Fig. 10). The presence of microplastics in the digestive tract indicates uptake of the particles from the water by *C. fluminea* and confirm the evidences discussed in section 5.3.1. As a non-selective filter feeder, *C. fluminea* is able to filter algae, bacteria and inert particles from the water column (Foe and Knight, 1986). Moreover, evidences from laboratorial and field studies indicate that *C. fluminea* ingests and accumulates microplastics (Guilhermino *et al*., 2018). In the present study, microplastics were also in *C. fluminea* gills, in good agreement with previous findings in the same species and other bivalves (Paul-Pont *et al*., 2016; Guilhermino *et al*., 2018). Moreover, it is possible that uptake and absorption of microplastics through the gills occurred, as previously suggested for very small plastic particles GESAMP (2015).

The concentrations of mercury in the body of bivalves are shown in Table 10. No significant differences in the mean of mercury concentration determined in bivalves shortly after field collection, after acclimation and in the controls of the bioassay were found (1-way ANOVA, $F_{(3, 11)} = 2.95$; $p > 0.05$). This result indicates that mercury levels did not change significantly during the acclimation period. It also shows that at the beginning of the bioassay animals had comparable levels of mercury, and thus, the observed differences could be attributed to exposure to different experimental treatments.

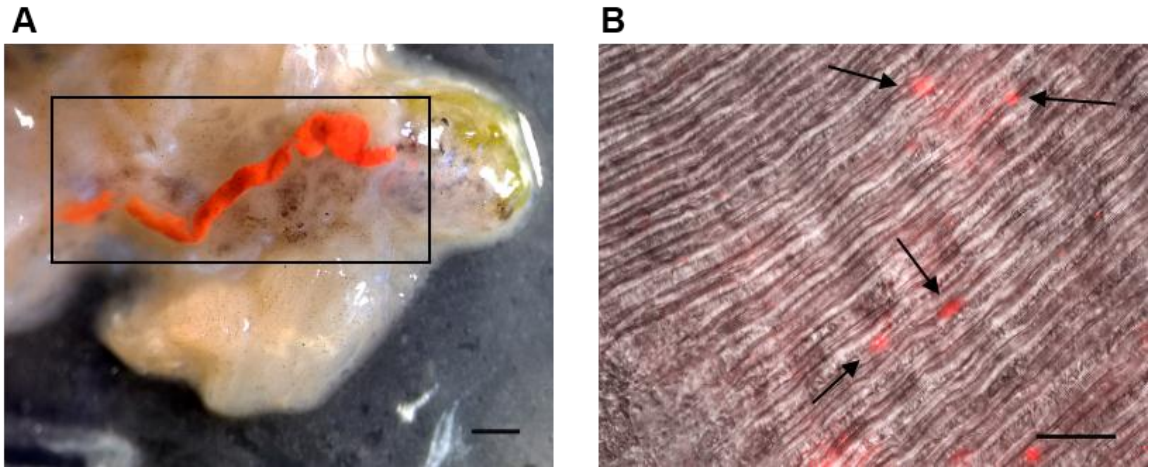

Fig. 10. Microplastic particles detected in the body of *Corbicula fluminea* exposed to microplastics alone for 8 days. A - Digestive tract (outlined in a box; scale bar = 10 mm) and B - Gill tissue (indicated by arrows; scale bar = 500 µm).

The mean concentration of mercury in the two control groups of the bioassay was 0.042 µg/g ww (Table 10). After 8 days of exposure to mercury alone, the mercury BFC was 55 indicating a high bioconcentration of mercury by *C. fluminea*. The BCF was higher (~2 folds) in bivalves of the treatment containing the mercury alone (55) than in bivalves exposed to the mixture (25), and a significant interaction between mercury and microplastics was found (3-way ANOVA, Table 10). These findings indicate that microplastics decreased the bioconcentration of mercury by *C. fluminea*. At least four hypotheses that are not mutually exclusive may be raised to explain this finding. First, decrease of mercury concentration in test media due to sedimentation of part of the microplastics with mercury bound, as suggested by the results of section 5.3.1, leading to a reduction of the bioavailability of the metal in test medium.

Table 10. Results of the three-way ANOVA performed to investigate the effects of mercury (Hg), microplastics (MP) and Recovery on Hg body concentrations of *Corbicula fluminea*. The Hg concentrations and the bioconcentration factors were determined in bivalves collected from the field, after 14 days of acclimation and after exposure to different treatments of the bioassay.

Condition	**Estimated exposure concentration (mg/L)**	**Hg body concentration (µg/g ww)**	**Bioconcentration factor**
Field	-	0.003 ± 0.004	
Acclimation	-	0.027 ± 0.004	
8-day control	0	0.039 ± 0.012	-
8-day MP	0	0.039 ± 0.012	-
8-day Hg	0.031 ± 0.014	1.75 ± 0.40	55
8-day Mixture	0.025 ± 0.018	0.67 ± 0.091	25
14-day control	0	0.045 ± 0.012	-
MP-Recovery	0	0.045 ± 0.012	-
Hg-Recovery	0.031 ± 0.014	0.55 ± 0.21	18
Mixture-Recovery	0.025 ± 0.018	0.43 ± 0.23	15

Factor	Level	Hg body concentration (µg/g ww)	3-way ANOVA
Hg	No Yes	0.04 ± 0.01 0.85 ± 0.59	$F_{(1, 23)}$ = 120; p = 0.000
MP	No Yes	0.60 ± 0.75 0.30 ± 0.297	$F_{(1, 23)}$ = 16.7; p = 0.001
Recovery	No Yes	0.62 ± 0.75 0.27 ± 0.27	$F_{(1 ,23)}$ = 23.5; p = 0.000
Hg x MP			$F_{(1, 23)}$ = 16.7; p = 0.001
Hg x Recovery			$F_{(1, 23)}$ = 24.3; p = 0.000
MP x Recovery			$F_{(1, 23)}$ = 10.7; p = 0.005
Hg x MP x Recovery			$F_{(1, 23)}$ = 10.7; p = 0.005

Although *C. fluminea* may uptake microplastics with mercury from the bottom of test beakers, likely it uptakes more through filtration of the water column. Second, when both mercury and microplastics are ingested from test media, part of the metal binds to the particles in *C.*

fluminea digestive tract and part of the microplastics with mercury adsorbed is eliminated. This process likely occurs because *C. fluminea* ingested microplastics (Fig. 10) and mercury likely adsorbs to the particles (section 5.3.1). Third, after absorption of both substances by *C. fluminea*, independently and/or bound, toxicological interactions occur increasing the metabolization and/or elimination of the metal, and/or decreasing its deposition in internal storage compartments. This is also a possibility because the toxicity of the mixture was lower than the toxicity induced by the substances alone (Fig. 11). Finally, the presence of microplastics in the gills may have decreased the filtration capacity leading to a reduction of mercury uptake. The decrease of FR observed in bivalves exposed to microplastics alone and in mixture (Fig. 11A) and the previous results of other authors showing than other microplastics negatively affected the FR in another bivalve species (Rist *et al*., 2016) provide support to this hypothesis.

The decrease of *C. fluminea* mercury BCF in the presence of microplastics found in the present study is in good agreement with comparable findings in *Dicentrarchus labrax* (Barboza *et al*., 2018b). At the end of the recovery period bivalves may not yet have reached the steady state in relation to mercury concentrations, thus the BCFs indicated should be regarded with caution. The mercury BCFs were considerably lower than those determined at the end of 8 days of exposure (Table 10). Moreover, they were comparable in bivalves of the mercury-recovery (18) and in those exposed to the mixture-recovery (15). Thus, after exposure, animals eliminated mercury from the body rapidly reaching similar concentrations after 6 days in clean medium despite having a considerable difference at the end of the mercury exposure. Moreover, 6 days were not enough to eliminate completely the metal from *C. fluminea* body. As mercury is very toxic to humans and occurs globally, this finding is of interest regarding human food safety, namely in relation to the depuration period that should be established before *C. fluminea* from wild populations could be consumed as food by humans.

5.3.3. Effects of microplastics, mercury and mixture in biomarkers and post-exposure recovery

The results of the 3-way ANOVA carried out with each biomarker data set are shown in Table 11. Exposure to microplastics (alone or in mixture) had a significant main effect on FR and GST activity. A significant main effect of Hg (alone or in mixture) was observed for all biomarkers except ODH. The recovery period had a significant main effect on the activities of

ChE, IDH and GST and on LPO levels. Significant interactions between two or among three factors were found for several biomarkers.

Table 11. Results of the three-way ANOVA of the biomarkers of *Corbicula fluminea* performed to investigate the effects of microplastics (MP), mercury (Hg) and recovery on: filtration rate (FR), cholinesterase enzymes (ChE) activity, NADP-dependent isocitrate dehydrogenase (IDH) activity, octopine dehydrogenase (ODH) activity, catalase (CAT) activity, glutathione reductase (GR) activity, glutathione peroxidase (GPx) activity, glutathione *S*-transferases (GST) activity and lipid peroxidation (LPO) levels. The significant level was 0.05.

		Biomarker								
Source of variation		FR	CHE	IDH	ODH	CAT	GR	GPx	GST	LPO
	df	(1, 48)	(1, 72)	(1, 72)	(1, 72)	(1, 72)	(1, 72)	(1, 72)	(1, 72)	(1, 72)
MP	F	110.9	1.628	0.094	0.644	0.857	1.990	0.024	19.128	0.570
	p	0.000	0.207	0.760	0.425	0.358	0.163	0.877	0.000	0.453
	df	(1, 48)	(1, 72)	(1, 72)	(1, 72)	(1, 72)	(1, 72)	(1, 72)	(1, 72)	(1, 72)
Hg	F	267.5	5.518	13.54	0.906	77.612	27.868	52.007	75.38	62.35
	p	0.000	0.022	0.000	0.345	0.000	0.000	0.000	0.000	0.000
	df	(1, 48)	(1, 72)	(1, 72)	(2, 105)	(1, 72)	(1, 72)	(1, 72)	(1, 72)	(1, 72)
Recovery	F	0.08	6.008	9.710	0.840	0.114	2.441	0.728	17.071	9.109
	p	0.927	0.017	0.004	0.363	0.737	0.123	0.380	0.000	0.004
	df	(1, 48)	(1, 72)	(1, 72)	(2, 105)	(1, 72)	(1, 72)	(1, 72)	(1, 72)	(1, 72)
MP x Hg	F	668	7.048	3.077	0.1191	0.010	1.640	1.368	41.37	56.037
	p	0.00	0.001	0.084	0.731	0.919	0.205	0.247	0.000	0.000
	df	(1, 48)	(1, 72)	(1, 72)	(1, 72)	(1, 72)	(1, 72)	(1, 72)	(1, 72)	(1, 72)
Hg x Recovery	F	0.961	0.052	0.365	0.670	0.045	0.617	8.013	0.145	0.145
	p	0.333	0.820	0.546	0.201	0.833	0.435	0.910	0.006	0.705
	df	(1, 48)	(1, 72)	(1, 72)	(1, 72)	(1, 105)	(1, 72)	(1, 72)	(1, 72)	(1, 72)
MP x Recovery	F	1.421	0.615	4.020	1.228	10.81	5.805	0.085	6.236	6.236
	p	0.240	0.430	0.049	0.272	0.002	0.019	0.722	0.001	0.015
	df	(1, 48)	(1, 72)	(1, 72)	(1, 72)	(1, 72)	(1, 72)	(1, 72)	(1, 72)	(1, 72)
MP x Hg x Recovery	F	0.098	0.448	1.622	0.486	0.070	1.150	0.869	6.311	6.311
	p	0.756	0.506	0.207	0.488	0.758	0.228	0.355	0.129	0.015

Overall, these results indicate that both microplastics and mercury caused toxic effects in bivalves, which suggest toxicological interactions between microplastics and mercury, and indicate that some post-exposure recovery occurred.

To go further on the effects caused by the tested substances and their potential toxicological interactions on each biomarker, the individual treatments were compared through 1-way

ANOVA (Table 12) and the Tukey's test, except for ODH because no significant differences in any factor and no significant interactions were found by 3-way ANOVA (Table 11). The results of FR, ChE, IDH, CAT, GR, GPx, and GST activities and LPO levels are sown in Figs. 11A to 11H, respectively. No significant differences between the two control groups were found for any parameter (Fig. 11). Thus, control animals were in good health conditions during all the exposure period and any differences between these and other groups were due to distinct treatments.

Table 12. Results of the one-way ANOVA carried out with the data of each biomarker to compare different experimental treatments. FR - Filtration rate; ChE - Cholinesterase enzymes activity; IDH - NADP-dependent isocitrate dehydrogenase activity; ODH - Octopine dehydrogenase activity; CAT - Catalase activity; GR - Glutathione reductase activity; GPx - Glutathione peroxidase activity; GST - Glutathione *S*-transferases activity; LPO - Lipid peroxidation levels. Df - Degrees of freedom.

Biomarker	df	F	p
FR	(7, 71)	150	0.000
ChE	(7, 71)	3.05	0.008
IDH	(7, 71)	4.56	0.000
ODH	(7, 71)	0.84	0.557
CAT	(7, 71)	12.5	0.000
GR	(7, 71)	6.30	0.000
GPx	(7, 71)	7.90	0.000
GST	(7, 71)	28.8	0.000
LPO	(7, 71)	2.01	0.000

In relation to the controls, bivalves exposed to the 8 day-microplastics treatment had significantly decreased FR (95% reduction, Fig. 11A) inhibition of ChE activity (15% inhibition, Fig. 11B) and increased LPO levels (~2 folds, Fig. 11H). FR reduction indicates that animals had a decreased capability of getting microalgae and other food items from the water column through filtration. Because such food source is important for *C. fluminea* (Foe and Kight, 1986), if not compensated by other food intake mechanisms, this may cause nutrients and oligo-elements depletion and decrease of energy.

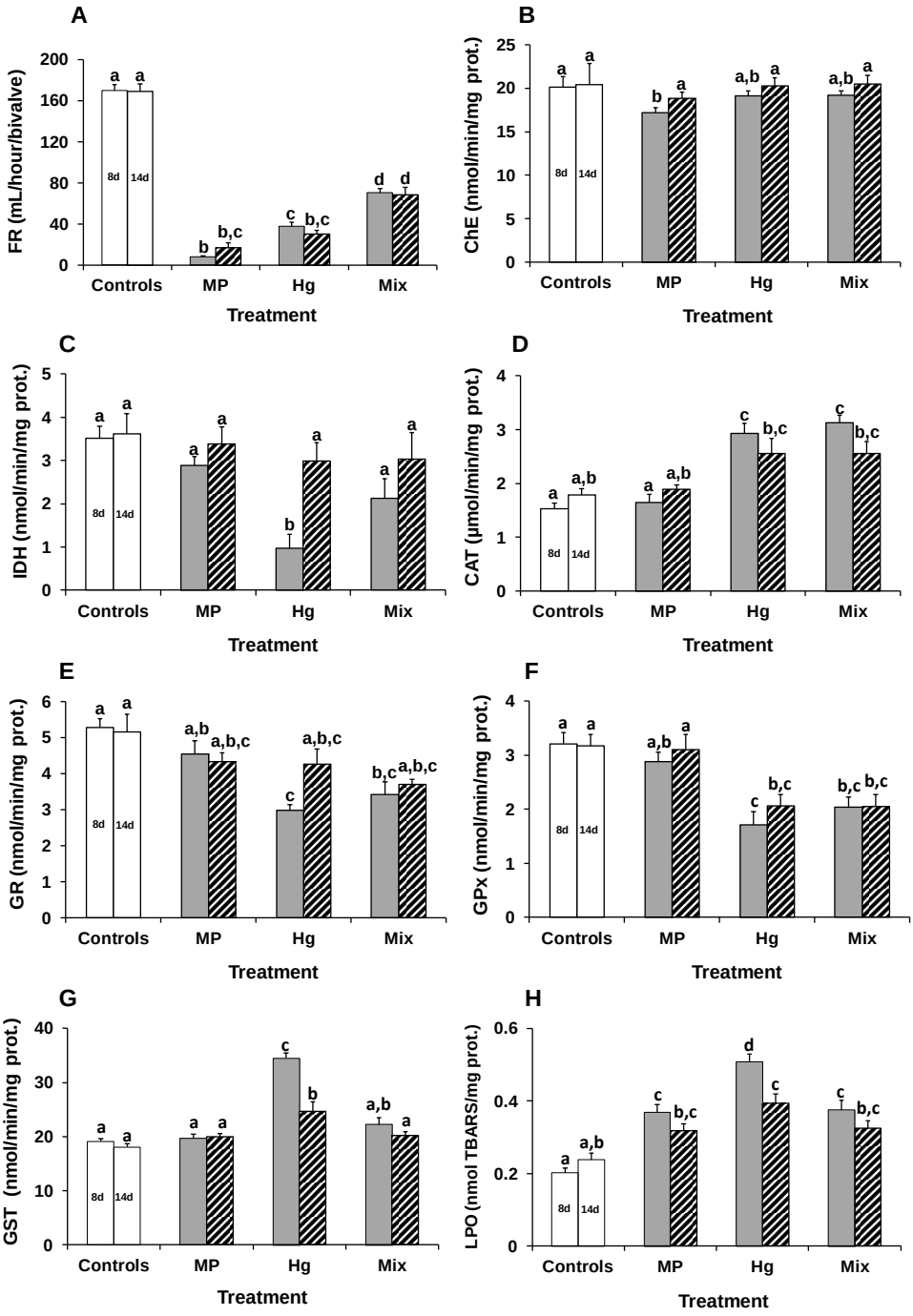

Fig. 11. Biomarkers determined in *Corbicula fluminea* after 8 days of exposure to microplastics (MP), mercury (Hg) and mixture (Mix) (grey bars) and after the post-exposure recovery period (striped bars). A - Filtration rate (FR), B - Cholinesterase enzymes (ChE) activity, C - NADP-dependent isocitrate dehydrogenase (IDH) activity, D - Catalase (CAT) activity, E - Glutathione reductase (GR) activity, F - Glutathione peroxidase (GPx) activity, G - Glutathione *S*-transferases (GST) activity and D - Lipid peroxidation (LPO) levels. Significant differences between treatments are identified by different letters above the bars (one-way ANOVA and the Tukey's test, $p \leq 0.05$).

In these conditions, animals likely will allocate the most part of the energy available to maintenance and repair instead of investing in growth and reproduction (Matzelle *et al*., 2014). Several factors may have caused the microplastics-induced FR reduction, mainly the presence of microplastics in the gills as previously discussed (section 5.3.2), the presence of microplastics in the digestive system and ChE inhibition. Microplastics in the digestive system may have induced false food satiation as previously suggested for *C. fluminea* and other bivalves (Guilhermino *et al*., 2018). False food satiation is a well known effect of microplastics that has been observed in several aquatic species (Farrel and Nelson, 2013). ChE inhibition may have also contributed to FR decrease, because the inhibition of the enzymatic activity determined in the adductor muscle may have caused neurotoxicity and neuromuscular disruption of cholinergic transmission. Such effects may negatively impact a wide range of physiological and biological processes in bivalves, including shell opening and closing regulation, control of muscles necessary to filtration, respiration, among several other functions (Rist *et al*., 2016).

Increased LPO levels indicate microplastics-induced oxidative stress and lipid oxidation damage, an effect that can have negative impact of several physiological processes. Microplastics-induced FR reduction, ChE inhibition, and oxidative stress and damage were found previously in bivalves exposed to different types of microplastics (Ribeiro *et al*. 2017; Guilhermino *et al*., 2018).

Bivalves of 8-day mercury treatment had significantly decreased FR (78% decrease, Fig. 11A), IDH activity (72% inhibition, Fig. 11C), GR activity (44% inhibition, Fig. 11E) and GPx activity (47% inhibition, Fig. 11F), and significantly increased CAT activity (~2 folds, Fig. 11D), GST activity (~2 folds, Fig. 11G) and LPO levels (2.5 folds, Fig. 11H). FR inhibition has consequences similar to those previously discussed for microplastics, and has been reported in bivalves exposed to mercury, such as *Perna perna* (Anandraj *et al.* (2002). IDH activity inhibition combined with FR decrease suggests decrease of internal oxygen levels and decrease of the use of anaerobic pathways of cellular energy production. Because IDH function is necessary to maintain the cell redox balance (Jo *et al*., 2001) its inhibition may also indicate decrease of the capability to deal with oxidative stress, as also suggested by the inhibition of two antioxidant enzymes (GR and GPx) activities. This is particularly critical because mercury induced oxidative stress as indicated by the significant increase of CAT and GST activities, and may have contributed to the failure of the antioxidant system to prevent oxidative damage to occur as indicated by the significant increase of LPO levels. Mercury-

induced enzymatic inhibition (including of IDH activity), oxidative stress and damage are well known effects of mercury that were previously reported in several bivalves including *C. fluminea* (Ahmad *et al*., 2011; Oliveira *et al*., 2015b).

Bivalves exposed for 8 days to the mixture and analysed immediately had significantly decreased FR (58% reduction, Fig. 11A), GR activity (35% inhibition, Fig. 11E), and increased CAT activity and LPO levels (~2 folds, Figs. 11D and 11H, respectively). Thus, as its individual components, the mixture also had negative effects on the FR, and caused oxidative stress and lipid peroxidation damage. However, the comparison of the effects caused by the mixture and its components individually, shows that the inhibition of FR caused by the mixture was significantly lower than the sum of the decrease caused by microplastics and mercury alone (Fig. 11A). Moreover, mercury alone induced significant IDH inhibition and GST induction, and microplastics alone inhibited ChE activity whereas such effects were not observed in animals exposed to the mixture. These differences indicate antagonism between microplastics and mercury in *C. fluminea* FR, IDH activity, ChE activity and GST activity. Binding of mercury to microplastics in test media may have contributed to the lower effects of the mixture in relation to the effects caused by mercury alone on some of the biomarkers. But it cannot explain other differences, such as the lack of significant ChE inhibition in animals exposed to the mixture, an effect that was induced by microplastics alone, and the lower FR decrease caused by the mixture than by microplastics alone. Thus, interactions between microplastics and mercury inside the animals resulting in antagonism must have occurred.

After 6 days of post-exposure recovery, bivalves of the microplastics-recovery treatment still had significant reduction of FR with no signs of significant recovery (Fig. 11A). However, bivalves had no significant differences in ChE activity (Fig. 11B) and in LPO levels (Fig. 11H) in relation to the 14-day control group, indicating recovery of these biomarkers. Animals exposed to the mercury-recovery treatment showed significant differences in relation to the 14-day control group in FR, CAT activity, GPx activity, GST activity and LPO levels (Figs. 11A, D, F, G and H, respectively), and no significant differences in IDH and GR activities (Figs. 11C and 11E, respectively). Thus, recovery in some biomarkers occurred but not in others.

5.4. Conclusions

Evidences of interactions between mercury and microplastics in test medium were found, suggesting adsorption of mercury to the microplastics tested. After 8 days of exposure, the mercury BCF was lower in the presence of microplastics than in the absence of the particles, indicating that microplastics decreased the bioconcentration of mercury by *C. fluminea*. After 8 days of exposure, microplastics and mercury, alone and in mixture, significantly decreased the FR and induced oxidative stress and lipid peroxidation damage in *C. fluminea*, indicating reduced individual fitness. Additionally, microplastics alone also caused ChE inhibition, indicating neurotoxicity. The effects caused by the mixture in several biomarkers were lower than the effects induced by the substances when tested alone, indicating antagonism between mercury and microplastics. Six days of post-exposure in clean media was not enough to eliminate completely the mercury from the body of the bivalves, either in the presence or absence of microplastics. Moreover, after this period, bivalves did not recover completely from the exposure to microplastics, mercury or their mixture. These findings have implications to animal, ecosystem and human health, including regarding human food safety. Moreover, they highlight the importance of further investigating the effects of microplastics-metal mixtures and the post-exposure recovery in aquatic animals.

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Supplementary material

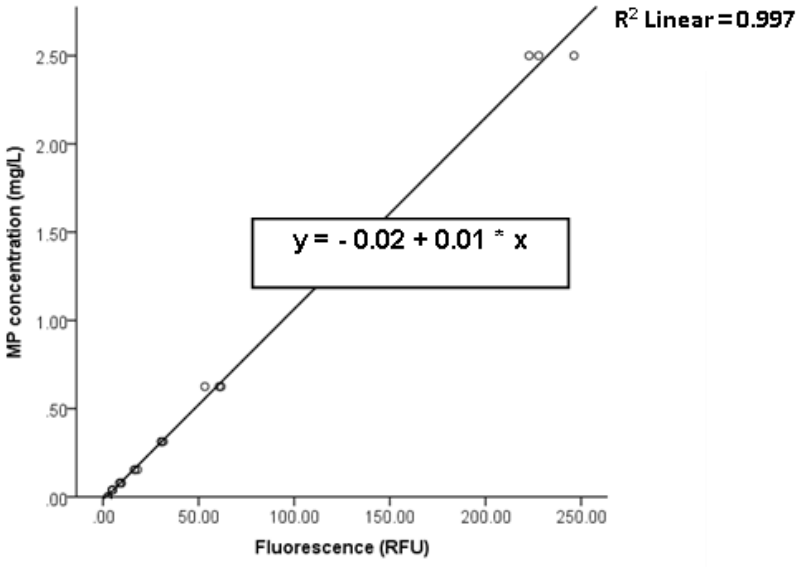

Fig. 12. Calibration curve of fluorescence *versus* concentration of microplastics (MP, mg/L) in clean medium, and the linear regression model: MP concentration = - 0.02 + 0.01 x fluorescence. RFU - Relative fluorescence units.

Table 13. Obtained and certified concentrations of mercury (Hg, µg/g, dry weight) in certified reference material (CRM) BCR 463 (mercury and methylmercury in tuna fish) and the respective recovery percentage.

CRM BCR 463	Hg concentration (µg/g, dry weight)
Certified	2.85 ± 0.16
Obtained	2.85 ± 0.16
Recovery	94%

CHAPTER VI

General discussion and concluding remarks

Bioinvasions are recognized as a major global environmental problem constituting a challenge for scientists, policy makers and the general public. In the present context of global climate changes, biological invasions became even more concerning. Thus, management efforts have been made in order to prevent, control and mitigate the negative impact of NIS. *C. fluminea* is one of the most prolific NIS in several aquatic ecosystems worldwide and deserves special attention given its negative ecological and socio-economic impacts. In this sense, investigating the effects of relevant environmental contaminants on this species is of major importance regarding the environmental and human health.

Mercury is a ubiquitous contaminant with high environmental persistence and toxicity to organisms and humans, and identified as a priority hazardous substance under the WFD (EC, 2008a), which means that, due to the risks it poses in environmental and human health their emissions or discharges must be phased out. It is a natural element and its environmental concentrations in certain areas are considerable increased due to anthropogenic activities, such as mining and several industrial processes (Barregard, 2008; Kerfoot *et al*., 2018). Despite the large amount of studies on the toxic effects of mercury over a wide range of aquatic species, more research regarding invasive species is needed since mercury is a common contaminant in several ecosystems that these species inhabit (Schmitt *et al*., 2011).

Microplastics are contaminants of emerging concern and considered a global problem due to their adverse effects on the environment, animal and human health (Santillo *et al*., 2017; Li *et al*., 2018). Additionally microplastics can interact with other contaminants, influencing their toxicity (Guilhermino *et al*., 2018).

Considering the above indicated major environmental paradigms, the present Thesis investigated the effects of mercury alone an in mixture with microplastics in one of the worst aquatic NIS (*C. fluminea*) and its capability of post-exposure recovery. The question of the sensitivity of this NIS in relation to one of its natural bivalve competitors with conservational interest (*A. anatina*) was also investigated.

Overall, the results of the experimental work carried out with *C. fluminea* specimens from wild populations of NW Portuguese estuaries contributed to increase the knowledge on the effects of mercury on *C. fluminea* through an integrative approach based on the assessment of changes at individual and sub-individual biological organization levels after different types of mercury exposures. Thus, mortality, the OCR and the FR were selected to investigate the effects of mercury on survival and on the individual fitness, respectively. The work also aimed an in-depth knowledge of the effects of mercury on different biochemical biomarkers that are

linked to important cellular functions including neurotransmission (ChE activity), aerobic and anaerobic energy production (IDH and ODH activities), respectively, and antioxidant defence system (CAT, GR, GPx and GST activities). The pertinence of their use was based in two aspects: first, they allow studying mechanisms of toxicity of chemical substances and secondly, changes at sub-individual levels are early warning signs of more severe forms of toxicity that may precede alterations at individual level, including impairment of essential functions for the survival and fitness of the organism (Boldina-Cosqueric *et al*., 2010).

In a first phase of the work (Chapter II), the suitable acclimation period to laboratory conditions before using *C. fluminea* from natural populations in bioassays based on the previously indicated sub-individual biomarkers was found to be 14 days, providing a basis to an effective use of these biomarkers as effect criteria in toxicity bioassays with *C. fluminea* from wild populations.

The work of the Chapter III provided new insights on the differences of sensitivity between *C. fluminea* and one of its native competitors with conservational interest (*A. anatina*) to mercury. The higher tolerance of *C. fluminea* to acute mercury exposure was evident from the lack of mortality of the species up to 500 µg/L of the metal, whereas a high mortality (72 h-LC_{50} = 49.6 µg/L) was observed in *A. anatina*. The induction of antioxidant mechanisms together with long-lasting periods of valve closure likely contributed to the high tolerance of *C. fluminea* to mercury. Environmental pollution is believed to modulate competition processes between NIS and native species, often acting in favour of NIS (Piola and Johsnton, 2008). Nevertheless, NIS are not always less sensitive to environmental contaminants than their native competitors, as shown in previous toxicity studies. For example, Faria *et al*. (2010) found that *C. fluminea*, despite showing a strong antioxidant response, had higher LPO levels than the native freshwater mussel *Psilunio litorallis* after transplantation to sites located next to mercury discharges from a chloralkali industry, suggesting that the NIS was more sensitive to this type of pollution than the native species in field conditions. Likewise, differences in sensitivity towards arsenic and mercury between the native *Ruditapes decussatus* and the exotic invasive *Ruditapes philippinarum* bivalves were found, with the former showing higher tolerance to the metals than the latter (Velez *et al*., 2016). However, higher tolerance to zinc of the exotic invasive *Sinanodonta woodiana* relatively to the native *A. anatina* was found (Bielen *et al*., 2016). Thus, the study of Chapter III provided a new contribution to the knowledge of interspecific differences of sensitivity to mercury between *C. fluminea* and one of its native competitors.

In the Chapter IV, the 14-day toxicity of mercury to *C. fluminea* specimens from two wild populations (those of the M-est and L-est) and their post-exposure recovery capability were compared using an approach that integrated individual (OCR) and sub-individual biomarkers (activities of ChE, IDH, ODH, CAT, GR, GPx and GST and LPO levels). The bivalves from M-est and L-est had different sensitivities and different post-exposure capabilities to mercury suggesting that the environmental conditions of the natural habitats of the populations influence the ability of the species to counteract the toxic effects of this metal, even after a 14-day period of acclimatization to laboratory conditions before the bioassay. The post-exposure recovery was a relevant aspect to investigate because the toxicant-induced effects may be transient or permanent after cessation of a contamination source. The recovery capability and delayed toxicity may have important implications, especially in a hypothetical acute pollution event in sites where *C. fluminea* inhabits.

The results of the work presented in Chapter V showed toxicological interactions between mercury and microplastics. Antagonism between these contaminants was found in FR, in ChE and GST activities and in LPO levels. Moreover, the bioconcentration of mercury was higher in animals exposed to mercury alone than in those exposed to the mixture. This was the first time that the effect of microplastics-mercury mixtures was investigated in *C. fluminea* and in a freshwater bivalve, thus increasing the knowledge on this subject, since studies on the toxicological interactions between microplastics and other environmental contaminants are still scarce (Barboza *et al*., 2018; Guilhermino *et al*., 2018). Although microplastics are recognized as an emergent pollutant of global concern and are abundant in freshwater systems (*e.g*. Rodrigues *et al*., 2018) there is no specific regulation regarding freshwaters (Brennholt *et al*., 2018). Thus it is necessary to draw attention to the effects of microplastics mixtures in species from wild populations inhabiting these ecosystems. This knowledge is also important regarding public health because *C. fluminea* is used for human consumption in some regions, and in many ecosystems mixtures of environmental contaminants are likely to occur.

Taking together the results of the different studies (Chapters III-V), one should emphasize the consistent effect that different exposures to mercury have had on some biomarkers, namely the inhibition of IDH and GR activities, induction of GST activity and increasing of LPO levels. This result indicates that mercury toxicity in *C. fluminea* involves alterations on the aerobic energy production pathway and on the antioxidant system. Moreover these responses were associated with marked reductions of OCR and FR indicating that the exposure to the metal decreased the individual fitness and health *status* of this species. Insights on the toxicity of

mercury to *C. fluminea* were accomplished by the studies carried out in the scope of the present Thesis. All the four specific questions were answered and provided important information regarding: i) the acclimation period that should be used before conducting toxicity bioassays with *C. fluminea* from wild populations based on certain sub-individual biomarkers (activities of ChE, IDH, ODH, CAT, GR, GPx and GST and LPO levels) (Chapter II); ii) the difference of sensitivity to mercury between *C. fluminea* and one of its native competitors with conservational interest (*A. anatina*) to mercury, namely the higher tolerance of the exotic species to this ubiquitous contaminant, a factor that may be advantageous to the NIS when in competition with the native species in mercury polluted ecosystems (Chapter III); iii) intraspecific differences in the sensitivity to mercury and post-exposure recovery between two *C. fluminea* wild populations related to environmental conditions of the populations natural habitats, highlighting the need of taking this factor into consideration in toxicity studies (Chapter IV); and iv) the influence of microplastics in the toxicity, post-exposure recovery and bioconcentration of mercury in *C. fluminea,* namely by decreasing the bioconcentration and toxicity of the metal, suggesting antagonism between these substances in this species (Chapter V) (Fig. 13).

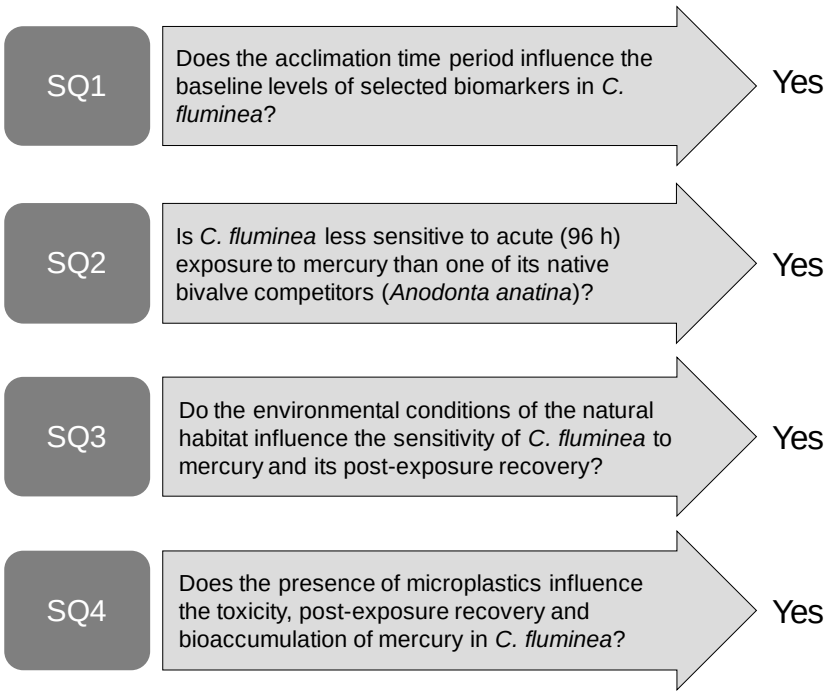

Fig. 13. Answers to the specific questions (SQ) formulated in the present Thesis (Chapter I).

Overall, the work carried out under the scope of this Thesis contributed to increase the knowledge on the effects of mercury on *C. fluminea* by giving information on the mechanisms involved on the toxicity of mercury on this species. Because environmental contamination has been pointed out as one of the factors that can influence the success of invasive species over the native ones, the findings of the present work may provide insights into the role that contamination may play in its invasive behaviour and colonization success, which is critical for support scientifically based management plans for *C. fluminea* in already invaded ecosystems.

Human consumption of *C. fluminea* in regions where this species is a NIS is neither a standard nor even regulated, thus there is little information regarding human health safety of this species. Therefore the findings of the present Thesis could also contribute for further knowledge in this area of expertise. For instance, the results on the bioaccumulation and elimination of mercury in the presence of widespread contaminants such as microplastics highlight the importance of determining effective depuration periods for mercury in the presence of other contaminants, which is a relevant aspect for the establishment of safety standards for human consumption. This knowledge is of critical importance regarding local unregulated consumption of *C. fluminea* (and the consequent need of regulatory measures) and also the evaluation of incentives to human consumption as a strategy for controlling this invasive species in heavily invaded ecosystems.

CHAPTER VII

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ANNEX I