UNIVERSIDADE DO ALGARVE

EFFECTS OF ACTIVE PHARMACEUTICAL INGREDIENTS IN MUSSEL *MYTILUS GALLOPROVINCIALIS*

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Resumo Geral

O aumento de produção e aplicação de compostos orgânicos resultou na sua deteção no ambiente. Estes compostos incluem disruptores endócrinos (também chamados xenoestrógenios, que afetam a regulação do sistema hormonal), pesticidas e produtos farmacêuticos e de higiene pessoal.

Tendo em conta, dados da União Europeia, existem mais de 3000 tipos de princípios ativos de origem farmacêutica aplicados na prática de medicina humana e veterinária. Os chamados ingredientes farmacêuticos ativos (do inglês, *active pharmaceutical ingredients* - APIs) são atualmente uma classe emergente de contaminantes ambientais com a capacidade de causar danos e efeitos inesperados em organismos aquáticos, nomeadamente em espécies de elevado interesse comercial (como peixes e moluscos).

Um dos fatores que desencadeou a necessidade de conhecer melhor esta problemática foi o aparecimento de fenómenos de *intersex* (mudança de sexo) em algumas espécies aquáticas que não podiam estar diretamente associados aos efeitos dos chamados contaminantes clássicos (i.e. metais, derivados de petróleo, pesticidas). Isto fomentou o desenvolvimento de novas tecnologias para a deteção e quantificação de fármacos na água e à necessidade de avaliar o efeito da exposição em diversas espécies a várias concentrações de produtos farmacêuticos de todas as classes terapêuticas.

Embora, o primeiro estudo que refere a incidência de fármacos no ambiente seja do final da década de 70, são ainda relativamente recentes os estudos nesta área. No entanto, uma enorme quantidade de APIs já foi detetada em afluentes e efluentes de estações de tratamento de águas residuais (ETARs), águas superficiais (fluviais e marinhas) e mais preocupantemente na água de abastecimento público de diversos países em concentrações na gama de ng a µg por Litro.

O principal problema do impacte de fármacos no ambiente refere-se à combinação de dois aspetos: a aplicação continuada e persistente pelas populações (com uma esperança média vida cada vez mais elevada) e ao fato da maioria destes compostos ser excretada praticamente na sua forma nativa ou na forma de metabolitos (por vezes, potencialmente mais tóxicos) que entram indiretamente (através de efluentes de hospitais e domésticos que não são eliminados nas ETARs) para os sistemas aquáticos.

Em Portugal, as ETARs estão somente capacitadas para a remoção de matéria orgânica e nalguns casos de nutrientes (fosfatos e nitratos). Além disso, não existe qualquer controlo relativamente à presença e posterior remoção de produtos de origem farmacêutica e higiene pessoal, o que pode levar a médio-longo prazo a sérios problemas ambientais e num cenário pior a perigo para a saúde pública.

Os mexilhões são mundialmente considerados excelentes instrumentos para avaliar os níveis de poluição marinha e estuarina, tendo sido selecionados como espécie sentinela em importantes programas internacionais de monitorização de contaminação aquática, nomeadamente o programa "Mussel Watch".

Tendo por base estas premissas, a presente tese teve como objetivos a deteção de APIs na água do Rio Arade (Portugal), o impacte do período estival na alteração das suas concentrações utilizando amostradores passivos de compostos orgânicos, POCIS (do inglês: *polar organic compounds integrative samplers*) e a avaliação dos efeitos numa espécie não-alvo, o mexilhão *Mytilus galloprovincialis* causados pela exposição a anti-inflamatórios não esteroides (AINEs) ibuprofeno (IBU) e diclofenac (DCF), bem como ao antidepressivo (inibidor seletivo da recaptação de serotonina - ISRS) fluoxetina (FLX) e suas misturas à qual também foi adicionado um contaminante clássico, o cobre (Cu). Para tal, foi efetuada uma análise de multibiomarcadores de *stress* oxidativo (atividade de enzimas antioxidantes como a: superóxido dismutase -SOD, catalase - CAT, glutationa redutase - GR e a enzima de fase II glutationa-S-transferase - GST), de dano - peroxidação lipídica (LPO), de efeito neurotóxico (através da atividade da enzima acetilcolinesterase - AChE) e de disrupção endócrina (pela medição de proteínas similares à vitelogenina através do método indireto de fosfatos-lábeis alcalinos - ALP) após a exposição desta espécie a concentrações ambientalmente relevantes.

Os principais resultados permitiram detetar a presença de 19 APIs no Rio Arade de diversas classes terapêuticas, cujas principais concentrações foram o alcaloíde cafeina, o antiasmático teofilina, AISE ibuprofeno e analgésico paracetamol. O impacte do período estival foi, no entanto, inconclusivo devido a alterações bastante significativas das concentrações nos meses amostrados.

Quanto à análise de biomarcadores, os resultados revelaram diferentes respostas para cada um dos APIs selecionados quer a nível de exposição individual ou na forma de misturas, sendo dependentes dos tecidos analisados e do tempo de exposição. São

também propostas diversas interações entre os diferentes componentes das misturas. Os resultados revelaram ainda o potencial de cada API para originar stress oxidativo, dano lipídico, efeito neurotóxico e disrupção endócrina, mesmo a concentrações extremamente baixas, numa espécie muito vulnerável à presença destes contaminantes emergentes dando enfoque à urgência da alteração e desenvolvimento de métodos preventivos à entrada destes compostos no ambiente aquático.

Palavras-chave: Compostos farmacêuticos ativos; ibuprofeno; diclofenac; fluoxetina; *Mytilus galloprovincialis*.

General Abstract

More than 3000 types of active pharmaceutical ingredients (APIs) are applied in Human and veterinary medicine practice. These compounds are considered an emergent class of environmental contaminants with the ability to cause damage and unexpected effects to aquatic organisms, namely in species of high commercial value. APIs are ubiquitous in the environment being frequently detected in influents and effluents of waste water treatment plants (WWTPs), surface waters and more distressingly in the public tap water in concentrations ranging from ng to $\mu g.L^{-1}$.

Considering these premises, the present thesis focused on APIs detection in the Arade river water, the impact of summer period in APIs' concentration alterations applying the passive sampler device, POCIS (polar organic compound integrative sampler), as well as, the assessment of the effects caused by non-steroidal anti-inflammatory drugs (NSAID) ibuprofen (IBU) and diclofenac (DCF) and antidepressant selective serotonin reuptake inhibitor (SSRI) fluoxetine as single and mixture exposures along with a classical contaminant copper (Cu) on a non-target species, mussel *Mytilus galloprovincialis*. For this purpose, a multibiomarker approach was applied namely including biomarkers of oxidative stress (antioxidant enzymes activities of superoxide dismutase – SOD, catalase – CAT, glutathione reductase – GR and Phase II glutathione-S-transferase), damage - lipid peroxidation (LPO), neurotoxic effects (through the activity of acetylcholinesterase enzyme - AChE) and endocrine disruption (through vitellogenin-like proteins measurement applying the indirect method of alkali-labile phosphate - ALP) after exposure of mussel species' to selected APIs at environmental relevant concentrations.

The main results highlighted the occurrence of 19 APIs in the river Arade from several distinct therapeutic classes. Stimulant caffeine, antiasthmatic theophylline, NSAID ibuprofen and analgesic paracetamol presented the highest concentrations. Summer impact was inconclusive due to each API transient concentration in each month.

The multibiomarker results revealed distinct responses towards each selected API (as single exposure or as mixtures) that were tissue and time dependent. Several multistressor interactions were proposed for each biomarker. The results also revealed

APIs potential to induce oxidative stress, LPO, neurotoxicity and endocrine disruption even at extremely low concentrations on a species extremely vulnerable to APIs presence highlighting the urgency on the development of methodologies able to prevent its entrance in the aquatic environment.

Keywords: Active pharmaceutical ingredients (APIs); ibuprofen; diclofenac; fluoxetine; *Mytilus galloprovincialis*

Index

Chapter 1

1 General Introduction ... 1
1.1 Historical Background ... 1
1.1.1 Pharmacy as an Industry ... 1
1.1.2 Worldwide Pharmaceutical Production and Consumption ... 2
1.1.3 Pharmacy and Pharmaceutical Products Consumption in Portugal ... 6
1.2 Pharmaceutical Products Usage ... 9
1.3 Pharmaceuticals and Personal Care Products (PPCPs) and the Environment ... 9
1.3.1 Drug Metabolism and Excretion ... 11
1.3.2 APIs Sources and Routes in the Environment ... 12
1.3.3 Fate of APIs in Waste Water Treatment Plants (WWTPs) ... 15
1.3.4 APIs Transformation and Degradation in the Environmental ... 17
1.3.5 Occurrence in the Aquatic Environment ... 19
1.4 Current Legislation, Environmental Risk Assessment (ERA) of APIs ... 21
1.4.1 "Green Chemistry" ... 22
1.5 Ecotoxicological Effects on Aquatic Organisms ... 25
1.5.1 Mixture Toxicity ... 26
1.6 Biomarkers ... 26
1.7 Oxidative Stress ... 27
1.7.1 Antioxidant Enzymes: Oxidative stress biomarkers ... 28
1.7.2 Lipid Peroxidation (LPO) ... 30
1.8 Neurotoxic Effects ... 31
1.8.1 Acetylcholinesterase (AChE) ... 31
1.9 Endocrine Disruption ... 31
1.10 Selected Species ... 32
1.10.1 *Mytilus galloprovincialis* (Lamark, 1819) (Figure 1.8) ... 32
1.11 Selected APIs for Exposure ... 33
1.11.1 Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) ... 33
1.11.2 Selective Serotonin Reuptake Inhibitors (SSRIs) ... 38
1.12 Copper (Cu) ... 41

1.13 Objectives ... 42
1.14 References ... 44

Chapter 2

2 Occurrence of APIs in the Aquatic System ... 57
2.1 Introduction ... 57
2.1.1 Active and Biological Sampling in Water Quality Monitoring ... 58
2.1.2 Passive Sampling in Water Quality Monitoring ... 59
2.1.3 POCIS Concept and Modeling ... 59
2.2 Materials and Methods ... 61
2.2.1 Sites Characterization ... 61
2.2.2 POCIS Field Deployment ... 63
2.3 POCIS Content Analysis ... 65
2.3.1 Materials and Chemicals ... 65
2.3.2 POCIS Extraction ... 65
2.3.3 Calculation of APIs Concentration ... 68
2.3.4 Statistical Analysis ... 69
2.4 Results ... 70
2.4.1 First Screen ... 70
2.4.2 Evolution of APIS Concentrations ... 73
2.4.3 Principal Component Analysis (PCA) ... 78
2.5 Discussion ... 79
2.5.1 Summer Impact in APIs Concentrations in Arade River ... 79
2.5.2 Comparison with other TWA Concentrations in Surface Waters ... 80
2.5.3 Arade River API Concentrations in Comparison with other Studies Applying Spot Sampling in Surface Waters ... 84
2.6 Conclusions ... 93
2.7 References ... 94

Chapter 3

3A. Non-steroidal Anti-Inflammatory Drug (NSAID) Ibuprofen Distresses Antioxidant Defense System in Mussel *Mytilus galloprovincialis* gills ... 100
3.1 Introduction ... 101
3.2 Materials and Methods ... 102
3.2.1 Chemicals ... 102

3.2.2 IBU Exposure Assay (250 ng.L^{-1}) 103
3.2.3 Condition Index 103
3.2.4 Tissue Preparation for Antioxidant Enzyme Activities Analysis 104
3.2.5 Antioxidant Enzyme Analysis 104
3.2.6 LPO Analysis 105
3.3 Statistical Analysis 105
3.4 Results 105
3.4.1 Condition Index 105
3.4.2 Antioxidant Enzymes 106
3.4.3 LPO 108
3.4.4 Principal Component Analysis (PCA) 109
3.5 Discussion 110
3.6 Conclusions 112
3.7 References 113

3B. Does non-steroidal anti-inflammatory (NSAID) ibuprofen induce antioxidant stress and endocrine disruption in mussel *Mytilus galloprovincialis*?

3.8 Introduction 117
3.9 Materials and Methods 123
3.9.1 Chemicals 123
3.9.2 IBU Exposure Assay (250 ng.L^{-1}) 124
3.9.3 Tissue Preparation for Antioxidant Enzyme Activities Analysis 124
3.9.4 Antioxidant Enzymes Analysis 124
3.9.5 LPO Analysis 125
3.9.6 ALP Analysis 125
3.9.7 Statistical Analysis 126
3.10 Results 126
3.10.1 Antioxidant Enzymes 126
3.10.2 LPO 129
3.10.3 PCA – Oxidative Stress Biomarkers 130
3.10.4 ALP 132
3.11 Discussion 133
3.11.1 Oxidative Stress 133
3.11.2 Endocrine Disruption 135

3.12 Conclusions 136
3.13 References 137

Chapter 4

4 Does Selective Serotonin Reuptake Inhibitor (SSRI) Fluoxetine Affect Mussel *Mytilus galloprovincialis*? 145
4.1 Introduction 146
4.2 Materials and Methods 152
4.2.1 Chemicals 152
4.2.2 Fluoxetine Exposure Assay (75 $ng.L^{-1}$) 153
4.2.3 Antioxidant Enzymes Analysis 154
4.2.4 LPO Analysis 154
4.2.5 AChE Analysis 155
4.2.6 ALP Analysis 155
4.2.7 Statistical Analysis 155
4.3 Results 156
4.3.1 CI 156
4.3.2 Antioxidant Enzymes 156
4.3.3 LPO 160
4.3.4 AChE 162
4.3.5 ALP 163
4.3.6 PCA in Gills and Digestive Gland 164
4.4 Discussion 167
4.4.1 Oxidative Stress 167
4.4.2 Neurotoxic Effect 168
4.4.3 Endocrine Disruption 169
4.5 Conclusions 170
4.6 References 171

Chapter 5

5 Effects of Non-Steroidal Anti-Inflammatory drug (NSAID) Diclofenac Exposure in Mussel *Mytilus galloprovincialis* 180
5.1 Introduction 181
5.2 Materials and Methods 186
5.2.1 Chemicals 186

5.2.2 Diclofenac Exposure Assay (250 ng.L^{-1}) 187
5.2.3 Antioxidant Enzymes Analysis 188
5.2.4 LPO Analysis 188
5.2.5 AChE Analysis 189
5.2.6 ALP Analysis 189
5.2.7 Statistical Analysis 189
5.3 Results 190
5.3.1 Condition index 190
5.3.2 Antioxidant Enzymes 190
5.3.3 LPO 195
5.3.4 AChE 197
5.3.5 ALP 198
5.3.6 PCA in Gills and Digestive Gland 199
5.4 Discussion 202
5.4.1 Oxidative Stress 202
5.4.2 Neurotoxic Effect and Endocrine Disruption 204
5.5 Conclusions 205
5.6 References 206

Chapter 6

6 Effect of APIs mixtures in mussel *M. galloprovincialis* through a multibiomarker approach 216
6.1 Introduction 217
6.2 Materials and Methods 222
6.2.1 Chemicals 222
6.2.2 APIs Mixtures Exposure Assay 223
6.2.3 Antioxidant Enzymes Analysis 224
6.2.4 LPO Analysis 225
6.2.5 AChE Analysis 225
6.2.6 ALP Analysis 225
6.2.7 Statistical Analysis 226
6.3 Results 226
6.3.1 Condition Index 226
6.3.2 Antioxidant Enzymes 226

6.3.3 LPO ... 231
6.3.4 AChE ... 233
6.3.5 ALP ... 234
6.3.6 PCA in Gills and Digestive Gland ... 236
6.4 Discussion ... 238
6.4.1 Mixture components interactions ... 242
6.4.2 CAT activity ... 242
6.4.3 GR activity ... 243
6.4.4 GST activity ... 244
6.4.5 LPO level ... 244
6.4.6 AChE activity ... 245
6.4.7 Vitellogenin-like proteins (ALP levels) ... 246
6.5 References ... 248

Chapter 7

7 General Discussion ... 254
7.1 Occurrence of APIs in the Aquatic System: Arade River (Portugal) ... 255
7.2 Effects of selected pharmaceuticals in mussel *M. galloprovincialis* ... 259
7.2.1 Effect of IBU exposure in *M. galloprovincialis* (Chapter 3) ... 260
7.2.2 Effect of FLX exposure in mussel *M. galloprovincialis* (Chapter 4) ... 262
7.2.3 Effect of NSAID DCF exposure in *M. galloprovincialis* (Chapter 5) ... 266
7.2.4 Effect of APIs mixtures exposure in *M. galloprovincialis* (Chapter 6) ... 268
7.3 Conclusions ... 274
7.4 Future perspectives ... 274
7.5 References ... 276

Figure Index

Chapter 1

Figure 1.1: Scheme of API pathway in the body. 11

Figure 1.2: APIs metabolic biotransformation pathways to increase polarity and excretion 12

Figure 1.3: Pathways of APIs from production to the aquatic environment 14

Figure 1.4: APIs metabolites and transformation products 18

Figure 1.5: Therapeutic classes present in the environment between 1997 and 2009 20

Figure 1.6: Phase I of the prioritization methodology proposal based on EMEA guidelines applied to French situation. 23

Figure 1.7: Phase 2 of the prioritization methodology proposal based on ecotoxicological, pharmacological and physico-chemical data. 24

Figure 1.8: Diagram of reactive oxygen (ROS) formation. 28

Figure 1.9: Antioxidant enzymatic reactions cascade. 30

Figure 1.10: Mussel *Mytilus galloprovincialis.* 32

Figure 1.11: NSAID pathway in humans. 35

Figure 1.12: IBU chemical structure.. 36

Figure 1.13: DCF chemical structure. 37

Figure 1.14: FLX chemical structure. 39

Figure 1.15: Fluoxetine pathway in human and mammals. 40

Chapter 2

Figure 2.1: Polar Organic Integrative Sampler (POCIS). 59

Figure 2.2: POCIS deployment sites and WWTPs in Arade river. 62

Figure 2.3: POCIS deployment in site 1 (up) and in site 2 (down). 63

Figure 2.4: POCIS deployment in triplicate and protective cage. 64

Figure 2.5: POCIS disassembling (up) and transferal of sorbent to SPE cartridge (down). 65

Figure 2.6: TWA concentrations ($ng.L^{-1}$) in two sites of the Arade river in 2009. 70

Figure 2.7: TWA concentrations (ng.L^{-1}) in two sites of the Arade river in 2009. 71

Figure 2.8: Percentage of APIs in Arade river in 2009 according to therapeutic classe 72

Figure 2.9: TWA concentrations (ng.L^{-1}) (average ± SD) of CAT and THEO in Arade river sites between August to October 2010 (n = 3)... 73

Figure 2.10: TWA concentrations (ng.L^{-1}) (average ± SD) of PAR, CAR, IBU, DCF, NAP and GEM in Arade river sites between August to October 2010 (n = 3). 74

Figure 2.11: TWA concentrations (ng.L^{-1}) (average ± SD) of NOR, FLX, AMI, DIA, ALP in Arade river sites between August to October 2010 (n = 3).. 75

Figure 2.12: Percentage of APIs in Arade river in 2010 according to therapeutic classes. 77

Figure 2.13: PCA representing 61% of total variance of targeted-analytes concentration integration between sites and time from Arade river 2010. 78

Chapter 3

Figure 3.1: Antioxidant enzyme activities (mean ± standard deviation) in two-week IBU exposed and controls *M. galloprovincialis* gills... 107

Figure 3.2: Lipid peroxidation (mean ± standard deviation) in two-week IBU exposed and controls *M. galloprovincialis* gills... 108

Figure 3.3: PCA corresponding to the integration of antioxidant enzymes SOD, CAT, GR, GST, lipid peroxidation (LPO) and condition index (CI) on total variance (84.5%) between treatments 109

Figure 3.4: Antioxidant enzyme activities (mean ± standard deviation) in *M. galloprovincialis* digestive gland non- and exposed to IBU for 15 days. 128

Figure 3.5: Lipid peroxidation (LPO) (mean ± standard deviation) in control and IBU exposed digestive gland of mussel *M. galloprovincialis* over 15 days. 129

Figure 3.6: Principal component analysis (PCA) integrating antioxidant enzymes (SOD, CAT, GR, and GST) and LPO on total variance (94.3%) between treatments 131

Chapter 4

Figure 4.1: Antioxidant enzymes activity (mean ± standard deviation) in control and FLX exposed *M. galloprovincialis* tissues.. 158

Figure 4.2: LPO (mean ± standard deviation) in control and FLX exposed *M. galloprovincialis* tissues. 161

Figure 4.3: AChE activity (mean ± standard deviation) in non- and exposed mussels' *M. galloprovincialis* gills to 75 ng.L^{-1} of FLX along 15 days. 162

Figure 4.4: ALP level (mean ± standard deviation) in non- and exposed mussel's gonads *M. galloprovincialis* to 75 ng.L^{-1} of FLX throughout 15 days 163

Figure 4.5: PCA equivalent to the integration (A) of SOD, CAT and GST, LPO, AChE and CI on 77% of total variance in gills; - (B) of SOD, CAT and GST, LPO and CI on 83% of total variance in digestive gland of mussel *M. galloprovincialis* between treatments. 165

Figure 4.6: PCA related to the integration of SOD, CAT and GST, LPO on 85% of total variance in mussels´ *M. galloprovincialis* tissues between treatments. 166

Chapter 5

Figure 5.1: Antioxidant enzyme activities (mean ± standard deviation) in controls and DCF exposed *M. galloprovincialis* tissues.. 192

Figure 5.2: LPO (mean ± standard deviation) in mussels *M. galloprovincialis* control and DCF-exposed gills (A) and digestive glands (B) over 15 days. 196

Figure 5.3: AChE activity (mean ± standard deviation) in control and exposed mussels *M. galloprovincialis* gills to 250 ng.L^{-1} of DCF during 15 days.. 197

Figure 5.4: ALP levels (mean ± standard deviation) in control and exposed mussels *M. galloprovincialis* sex-differentiated gonads to 250 ng.L^{-1} of DCF during 15 days...... 198

Figure 5.5: PCA concerning (A) SOD, CAT, GR, GST, AChE activities, LPO levels and CI on 81% of total variance in *M. galloprovincialis* gills (○– gills controls, ● – DCF exposed gills) - (B) SOD, CAT, GR, GST activities, LPO levels and CI on 85% of total variance in digestive gland (♦ – digestive gland controls, ◊ – DCF exposed digestive gland) between groups over exposure time (days: 0, 3, 7 and 15)................ 200

Figure 5.6: PCA representing 82% of total variance of tissue-specific integration of antioxidant enzyme activities (SOD, CAT, GR and GST) and LPO levels between controls and DCF exposed mussels *M. galloprovincialis* (● – gills control, ○ – DCF exposed-gills, ♦– digestive gland controls and ◊ – DCF exposed-digestive gland) between groups over exposure time (days: 0, 3, 7 and 15) 201

Chapter 6

Figure 6.1: Antioxidant enzymes activities (mean ± standard deviation) in controls, mixtures 1 and 2 exposure of *M. galloprovincialis* tissues. SOD activity in gills (A) and digestive gland (B); CAT activity in gills (C) and digestive gland (D). Different letters express significant differences and bars signaled by an * express significant differences between females and males on respective day ($p < 0.05$). Note: Figure continues in the next page.. 228

Figure 6.2: LPO (mean ± standard deviation) in mussel *M. galloprovincialis* controls, mixtures 1 and 2 exposure in gills (A) and digestive glands (B) over 15 days. Different letters are significantly different and bars signaled by an * express significant differences between tissues on respective day ($p < 0.05$). 232

Figure 6.3: AChE activity (mean ± standard deviation) in controls, mixtures 1 and 2 exposed *M. galloprovincialis* gills during 15 days. Different letters express significant differences ($p < 0.05$). 233

Figure 6.4: ALP levels (mean ± standard deviation) in controls, mixture 1 and mixture 2 exposed *M. galloprovincialis* sex-differentiated gonads (A – females, B – males) during 15 days. Different letters express significant differences and bars signaled by an * express significant differences between females and males on respective day ($p < 0.05$). 235

Figure 6.5: PCA (A) represents 72% of total variance (TVar) of biomarkers (SOD, CAT, GR, GST, AChE, LPO and CI) integration in gills; PCA (B) represents 78% of TVar of biomarkers (SOD, CAT, GR, GST, LPO and CI) in digestive gland; PCA (C) represents 68 % of TVar of tissue-specific integration of biomarkers (SOD, CAT, GR, GST, LPO levels and CI) between controls and mixtures (1 and 2) exposed mussels *M. galloprovincialis* (○ - gills controls, ● – MIX 1-exposed gills, ● - MIX 2-exposed gills; ◊ – digestive gland controls, ♦ - MIX 1-exposed digestive glands, ♦ - MIX 2 exposed digestive glands) during exposure time (days: 0, 3, 7 and 15). 237

Figure 6.6: PCA (A and B) represents 70 and 80% of TVar of tissue-specific integration biomarkers (SOD, CAT, GST, LPO) in mixtures and single API exposed mussels *M. galloprovincialis* gills (A) and digestive gland (B) in relation to each time-control (● – IBU exposed gills, ● –DCF exposed gills ● – FLX exposed gills, ● – MIX 1-exposed gills, ● - MIX 2-exposed gills; ◊ – digestive gland controls, ♦ - IBU-exposed digestive glands, ♦ - DCF-exposed digestive glands, ♦ - FLX-exposed digestive glands ♦ - MIX 1 exposed digestive gland, ♦ - MIX 2 exposed digestive glands) during exposure time (0, 3, 7 and 15 days). 241

Table Index

Chapter 1

Table 1.1: World top pharmaceutical companies by value of sales in 2001 to 2010 (adapted from WHO, 2004; IMS Health, 2011) 2
Table 1.2: Top 10 pharmaceutical markets in the world between 2000 and 2010 with projection for 2015 (adapted from WHO, 2004; IMS Health, 2011) 3
Table 1.3: Top-20 most sold therapeutic classes in 2010 in the world (adapted from IMS Health, 2011) 4
Table 1.4: Top-20 most sold pharmaceutical product brands in the world (adapted from IMS Health, 2010) 5
Table 1.5: Portugal in World medicine situation in 2000 (WHO, 2004) 6
Table 1.6: Evolution of the top 50 active substances with highest number of packages in the Portuguese National Health Service from 2006 to 2009 (adapted from INFARMED, 2007; 2010) 7
Table 1.7: Chemical properties of selected pharmaceuticals: IBU, DCF and FLX (adapted from Tixier *et al.*, 2003 and retrieved online: DrugBank; ChemID plus Advanced) 33

Chapter 2

Table 2.1: Abiotic parameters measurement per site per year 64
Table 2.2: Targeted-APIs 66
Table 2.3: Type of ESI ionization and respective internal standard used. 67
Table 2.4: TWA concentrations of commonly detected APIs in surface waters using POCIS 82
Table 2.5: Maximum concentrations of the commonly detected APIs in surface waters collected by spot sampling 86

Chapter 3

Table 3.1: Condition Index (%) (mean ± standard deviation) of *M. galloprovincialis* non- and exposed to IBU for 2 weeks. Different letters express significant differences ($p < 0.05$) 105
Table 3.2: Occurrence of NSAID IBU ($ng.L^{-1}$) in worldwide surface and river water 118
Table 3.3: Ecotoxicological effects of APIs in mussel species 121
Table 3.4: ALP concentrations in sex-differentiated *M. galloprovincialis* gonads exposed to 250 $ng.L^{-1}$ of IBU for two weeks 132

Chapter 4

Table 4.1: Concentrations of FLX in the aquatic environment 147
Table 4.2: FLX effects in aquatic molluscs and fish 149

Chapter 5

Table 5.1: DCF concentrations ($ng.L^{-1}$) in the aquatic system 183
Table 5.2: DCF effects in aquatic species 184
Table 5.3: Pearson correlations between biomarkers in gills (G), digestive gland (DG) and sex-differentiated gonads (F or M) 194

Chapter 6

Table 6.1: Effects of APIs mixtures in aquatic organisms 219

Chapter 7

Table 7.1: Interaction between mixture components for each biomarker 270

Abbreviations/Acronyms:

4-HNE	(2E)-4-hydroxy-2-nonenal
5-HT	Serotonin (5-hydroxytrypamine)
AA	Arachidonic acid
ACh	Acetylcholine
AChE	Acetylcholinesterase
ALP	Alkali-labile phosphate
ANOVA	Analysis of variance
API	Active pharmaceutical ingredient
BPA	Bisphenol A
BSA	Bovine albumin serum
CA	Concentration addition
CA-HA	Carboxyhydratropic acid
CA-IBU	Carboxyibuprofen
cAMP	Cyclic adenosine monophosphate
CAS	Chemical abstracts service registration number
CAT	Catalase
CDNB	1-chloro-2,4,- dinitrobenzene
CERC	Columbia Environmental Research Center
CI	Condition index
COX	Cyclooxygenase
Cu	Copper
CYP P450	Cytochrome P450 superfamily
DBF	Phase I dibenzylflourescein dealkylase ()
DGPA	Direcção Geral das Pescas e Aquicultura
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DOC	Dissolved organic carbon
DOI	Digital object identifier
DTPA	Diethylenetriaminepentaacetic acid dianhydride
DTT	1,4-dithiothreitol
E2	17-estradiol
EC_{50}	Half maximal effective concentration
ED	Endocrine disruption
EDCs	Endocrine disruptor compounds
EDTA	Ethylenediaminetetraacetic acid
EEA	European Environment Agency
EMEA	European Medicine Evaluation Agency
EPA	United States Environment Protection Agency
ERA	Environmental risk assessment
ESI	Electrospray ionization
EST	Estrogen sulfotransferase
GABA	γ-aminobutyric acid
GC	Gas chromatography
GC-MS	Gas-chromatography-mass spectrometry

GHT	Butylated hydroxytoluene
GMPs	Good manufacturing practice
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione (reduced form)
GSSG	Glutathione (oxidized form)
GST	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide
HLB	Hydrophilic-lipophilic balanced
HPLC	High performance liquid chromatography
HRT	Hydraulic retention time
IA	Independent action
IC_{50}	Half maximal inhibitory concentration
INFARMED	Autoridade Nacional do Medicamento e
IPCS	International Program on Chemical Safety
KCl	Potassium chloride
LC-MS-MS	Liquid chromatography-mass spectrometry
LC_{50}	Lethal concentration (50%)
LC-ESI-MS-MS	Liquid chromatography electrospray ionization tandem mass
LC-Q-TOF-MS	Ultra-performance liquid chromatography–quadrupole–time-of-
LH	Polyunsaturated lipid
LLE	Liquid-liquid extraction
LOEC	Lowest observed effect concentration
Log K_{ow}	Octanol/water partition coefficient (log)
LOOH	Lipid hydroperoxide
LOX	Lipoxygenase
LPO	Lipid peroxidation
LPTC	Laboratoire de Physico- et Toxico-Chimie de l'Environnement
MCX	Mixed-mode cationic exchange
MDA	Malondialdehyde 1.1.3.3. tetramethoxypropane
MoA	Mode of action
NaCl	Sodium chloride
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	$NADP^+$ (reduced form)
NaOH	Sodium hydroxide
NOEC	No observed effect concentration
NSAID	Non-steroidal anti-inflammatory drug
$O_2^{\bullet -}$	Superoxide anion radical
OCT	Over the counter medicines
OECD	Organization for Economic Co-Operation and Development
$OH^{\bullet}$	Hydroxyl radical
OH-IBU	Hydroxyibuprofen
PAHs	Polycyclic aromatic hydrocarbons
PCA	Principal component analysis
PEC	Predicted environmental concentration
PFK	Glycolytic enzymes phosphofructokinase
PGs	Prostaglandins
PhAC	Pharmaceutically-active compounds

PK	Pyruvate kinase
pKa	$-\log_{10}$ of the acid dissociation constant of a solution
PKA	Protein kinase A
PLHC-1	Fish hepatoma cell line
PMID	PubMed identifier
PNEC	Predicted no effect concentration
PO_4	Phosphate
POCIS	Polar organic integrative sampler
POCs	Polar organic compounds
POM	Prescription only medicines
POPs	Persistent organic pollutants
PPCPs	Pharmaceuticals and personal care products
PRCs	Performance reference compounds
PS-DVB	Polystyrene-divinylbenzene
PST	Phenol sulfotransferase
RBC	Rotating biological contactors
RE	Removal rate
ROS	Reactive oxygen species
Rs	Sampling rate
SD	Standard deviation
SOD	Superoxide dismutase
SPE	Solid phase extraction
SPMD	Semipermeable membrane device
SPME	Solid phase microextraction
SRT	Solid retention time
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressants
TCA	Trichloroacetic acid
$ThCO_2$	Theoretical carbon dioxide production
ThOD	Theoretical oxygen demand
Tvar	Total Variance
TWA	Time-weighted average
UDP	Uridine diphosphate
UGT	UDP-dependent glucuronosyl transferase
UV	Ultra violet
Vn	Vitellin
Vtg	Vitellogenin
WHO	World Health Organization
WWTP	Waste water treatment plant

Active pharmaceutical ingredients (APIs):

EE2	17 α-ethynylestradiol	– contraceptive
ALP	alprazolam	– anxiolytic and sedative action
AMI	amitriptyline	– TCA antidepressant agent
ASA	acetylsalicylic acid	– anti-inflammatory and antipyretic agent
ATE	atenolol	– cardioselective β adrenergic blocker
ATO	atorvastatin	– statins (for lowering cholesterol)
BEZ	bezafibrate	– fibrate, antilipemic agent (for lowering cholesterol)
BRO	bromazepam	– anxiolytic action
BUP	bupropion	– unicyclic, aminoketone antidepressant agent
CAF	caffeine	– central nervous system stimulant
CAP	captopril	– inhibitor of angiotensin converting enzyme (ACE) (treatment of hypertension)
CAR	carbamazepine	– anticonvulsant
CIP	ciprofloxacin	– antibiotic
CLA	clofibric acid	– fibrate, lipid lowering agent
CLE	clenbuterol	– antiasthmatic bronchodilator
CYC	cyclophosphamide	– chemotherapy drug
DCF	diclofenac	– NSAID
DIA	diazepam	– anxiolytic, sedative, anticonvulsant
DOX	doxepin	– TCA antidepressant agent
FEN	fenofibrate	– fibrate, antilipemic agent
FLU	fluvoxamine	– SSRI antidepressant agent
FLX	fluoxetine	– SSRI antidepressant agent
FUR	furosemide	– diuretic (treatment of edema and chronic renal insufficiency)
GEM	gemfibrozil	– fibrate, lipid-regulating agent
HYD	hydrochlorothiazide	– thiazide diuretic (treatment of hypertension)
IBU	ibuprofen	– NSAID
IMI	imipramine	– TCA antidepressant agent
KET	ketoprofen	– NSAID
LEV	levofloxacin	– fluoroquinolone antibacterial agent
LIN	lincomycin	– antibiotic
MET	metoprolol	– cardioselective β1-adrenergic blocker
METF	metformin	– antihyperglycemic agent (treating non-insulin-dependent diabetes mellitus)
NAP	naproxen	– NSAID
NOR	nordiazepam	– anxiolytic
NOV	novobiocin	– antibiotic
OFL	ofloxacin	– fluoroquinolone antibacterial agent
OXY	oxytetracycline	– antibiotic
PAR	paracetamol/ acetaminophen	– analgesic and antipyretic agent
PAX	paroxetine	– SSRI
PRO	propanolol	– non-cardioselective β adrenergic antagonist (treatment of hypertension)
RAN	ranitidine	– non-imidazole blocker (treatment of

			gastrointestinal ulcers)
SAL	salbutamol	–	β2-adrenergic receptor agonist (treatment of asthma)
SER	sertraline	–	SSRI antidepressant agent
SIM	simvastine	–	hydroxymethylglutaryl CoA reductase inhibitor (rate-limiting enzyme in cholesterol biosynthesis)
SUL	sulfamethoxazole	–	bacteriostatic antibacterial agent
SULF	sulfapyridine	–	antibacterial agent
TER	terbutaline	–	antiasthmatic bronchodilator
THEO	theophylline	–	antiasthmatic bronchodilator
TRIC	triclosan	–	antibacterial and antifungal agent
TRIM	trimethoprim	–	antibiotic/antibacterial agent
VEN	venlafaxine	–	SSRI antidepressant agent

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Chapter 1

General Introduction

1 General Introduction

1.1 Historical Background

The oldest pharmaceutical document known reports how, perhaps 4000 years ago, Sumerians prepared some curative drugs. Illness was thought as a divine punishment and healing as the purification. That same concept was parallel throughout centuries (Kremers *et al.*, 1986). Only on the 5th century B.C. Hippocrates and his theory of "The Four Humors" changed that generalized view; the main basis for health was owed to the correct balance between blood, phlegm, yellow and black bile. Following Hippocrates stepping stone in Medicine, Galen also known as "father of Pharmacy", was the first to test the action of drugs associated to each specific humors' imbalance. Galenic concepts prevailed until the 16th century, when Paracelsus challenged them, announcing that illness actually resulted from outside body agents' attacks. Since then, the internal use of chemical remedies was made a matter of principle and study (Kremers *et al.*, 1986).

1.1.1 Pharmacy as an Industry

In the period between 1850 and 1914 as chemists enhanced the extent of their operations the retailing sector also expanded noticeably. By then, the use of the term "pharmaceutical" (from the Greek φάρμακον 'pharmakos' = drug) was widespread, and Pharmacy began to be seen as a prospective industry. Nowadays, the modern pharmaceutical industry has a high-technological image being one of the most prosperous economic sectors of the world (Tweendale, 2003). Pharmaceutical products regard a group of bioactive compounds with chemically complex molecular structures (which the key component is the active pharmaceutical ingredient - API) used for the diagnosis, treatment, mitigation or prevention of diseases or health conditions (Anderson *et al.*, 2004; Kümmerer, 2004). These products are generally categorized according to their therapeutic purpose (e.g. analgesics, antiasthmatics, antibiotics, antidepressants, antidiabetics, antiepileptics, antihypertensives, antilipidemics, antipsychotics, anti-ulcerants, autoimmune agents, non- and narcotics oncologics, etc.) see examples in Tables 1.3., 1.4 and 1.6 and from then on classified as prescription only medicines (POM) [requiring physician's instructions] or as "over the counter medicines" (OTC) being bought without any restriction (Anderson *et al.*, 2004; Kümmerer, 2004).

1.1.2 Worldwide Pharmaceutical Production and Consumption

Most pharmaceutical production is concentrated in industrialized countries in which two-thirds of the value of all medicines produced is accounted for by few transnational corporations headquartered in USA, UK, Switzerland, Germany, France and Japan (WHO, 2004; IMS Health, 2011) (Table 1.1).

Table 1.1: World top pharmaceutical companies by value of sales in 2001 to 2010 (adapted from WHO, 2004; IMS Health, 2011)

Corporation ranking (2001)		Corporation ranking (2010)
1. Pfizer (USA)	=	1. Pfizer (USA)
2. GlaxoSmithKline (UK/USA)	↓	2. Novartis (Switzerland)
3. Merck & Co. (USA)	=	3. Merck & Co. (USA)
4. AstraZeneca (Sweden/UK)	↓	4. Sanofi-Aventis (France)
5. Aventis (France/Germany)	↓	5. AstraZeneca (Sweden/UK)
6. Bristol-Myers Squibb (USA)	↓	6. GlaxoSmithKline (UK/USA)
7. Johnson & Johnson (USA)	↓	7. Roche (Switzerland)
8. Novartis (Switzerland)	↑	8. Johnson & Johnson (USA)
9. Upjohn/Pharmacia (USA)	↓	9. Abbott (USA)
10. Wyeth/American Home Products (USA)	↓	10. Eli Lilly (USA)
11. Eli Lilly (USA)	↑	11. Teva (Israel)
12. Roche (Switzerland)	↑	12. Bayer (Germany)
13. Bayer (Germany)	↑	13. Amgen (USA)
14. Schering-Plough (USA)	↓	14. Bristol-Myers Squibb (USA)
15. Abbott (USA)	↑	15. Boehring Ingel (Germany)
16. Takeda (Japan)	=	16. Takeda (Japan)

These countries are simultaneously the biggest individual exporters and importers of pharmaceutical products (WHO, 2004). The pharmaceutical consumption is measured by the country's pharmaceutical production value plus its import minus its export value (WHO, 2004). In terms of world pharmaceutical consumption from 2000 to 2010, USA clearly led global market sales (Table 1.2) (doubling from 150 to 300 billion US$ which worldwide corresponded from approximately 300 billion to over 800 billion US$ (10% of which were "over-the-counter" non-prescription drugs) (WHO, 2004; IMS Health, 2011) yet the global pharmaceutical market growth in average from 9% in 2003 to 4.1%

in 2010. This growth decrease tendency corresponded mostly to Japanese and North American market growth decline, which nevertheless was counter-balanced by the increment of Asian, African and South American countries markets (IMS Health, 2011).

Table 1.2: Top 10 pharmaceutical markets in the world between 2000 and 2010 with projection for 2015 (adapted from WHO, 2004; IMS Health, 2011)

2000	2005	2010	2015
1. USA	1. USA	1. USA	1. USA
2. Japan	2. Japan	2. Japan	2. Japan
3. France	3. France	3. China	3. China
4. Germany	4. Germany	4. Germany	4. Germany
5. UK	5. Italy	5. France	5. France
6. Italy	6. UK	6. Italy	6. Brazil
7. Spain	7. Spain	7. Brazil	7. Italy
8. Canada	8. Canada	8. Spain	8. India
9. Brazil	9. China	9. Canada	9. Spain
10. Mexico	10. Brazil	10. UK	10. Russia

In terms of total sales revenue of global pharmaceutical products in 2010, over 50% of global sales were represented by the top 20 therapeutic classes led by oncologic products (total 56 billion US$) (Table 1.3), nevertheless Lipitor® an anti-lipidemic (a.k.a. lipid-regulator) marketed by Pfizer was the world's best-seller pharmaceutical product brand engrossing 13 billion US$ in that year alone (IMS Health, 2011) (Table 1.4).

Table 1.3: Top-20 most sold therapeutic classes in 2010 in the world (adapted from IMS Health, 2011)

Therapeutic class	Billion US $
1. Oncologics	55972
2. Lipid Regulators	36400
3. Antiasthmatic/Respiratory Agents	35926
4. Antidiabetics	34429
5. Anti-ulcerants	27972
6. Antihypertensive/Angiotensin II Antagonists	26630
7. Antipsychotics	25412
8. Autoimmune agents	20710
9. Antidepressants	20216
10. HIV antivirals	15432
11. Platelet aggregation inhibitors	15244
12. Vitamins and minerals	12971
13. Antiepileptics	12553
14. Narcotic analgesics	12011
15. Antibiotics	11466
16. Non-narcotic analgesics	10986
17. Vaccines	10972
18. Erythropoietins	10596
19. Anti-rheumatics, non-steroidal	10152
20. Multiple sclerosis	9844
Global Market	791449

Table 1.4: Top-20 most sold pharmaceutical product brands in the world (adapted from IMS Health, 2010)

Pharmaceutical	Therapeutic class	Corporation	Billion US$
LIPITOR	Lipid regulator	Pfizer	12.6
PLAVIX	Platelet aggregation inhibitor	Bristol-Myers Squibb	8.8
SERETIDE	Antiasthmatic/Respiratory agent	GlaxoSmithKline	8.5
NEXIUM	Antiulcerants	AstraZeneca	8.4
SEROQUEL	Antipsychotic	AstraZeneca	6.8
CRESTOR	Lipid regulator	AstraZeneca	6.8
ENBREL	Autoimmune agent	Amgen and Pfizer	6.2
REMICADE	Autoimmune agent	Merck & Co.	6.0
HUMIRA	Autoimmune agent	Abbot	6.0
ZYPREXA	Antipsychotic	Eli Lilly & Co.	5.7
AVASTIN	Oncologics	Roche	5.5
SINGULAIR	Antiasthmatic/Respiratory agent	Merck & Co.	5.5
ABILIFY	Antipsychotics/antidepressant	Bristol-Myers Squibb	5.4
MABTHERA	Oncologics	Roche	5.0
LANTUS	Antidiabetic	Sanofi-Aventis	4.7
ARICEPT	Acetylcholinesterase inhibitor	Pfizer	4.4
ACTOS	Antidiabetics	Takeda	4.3
LOVENOX	Anticoagulant/Blood thinner	Sanofi	4.3
HERCEPTIN	Oncologics	Roche	4.2
DIOVAN	Antihypertensive	Novartis	4.2

1.1.3 Pharmacy and Pharmaceutical Products Consumption in Portugal

In Portugal, the pharmaceutical industry began at the end 19th century. Later on, the Great War recession required a higher effort of pharmaceutical production [since the majority of the pharmaceutical products came from Germany] forcing the emergence of subsidiary industries and new laboratories (Sousa Dias, 2008). Nowadays, the total market of pharmaceutical specialties sales reports an income of more than 3 billion € each year, corresponding to over 2 % of Portugal's GDP (INFARMED, 2006; 2009). Portuguese available data in terms of production, trade, total sales and expenditure in pharmaceuticals in 2000 is represented in Table 1.5 (WHO, 2004), while the top 50 active substances in Portugal are given in Table 1.6 comprising between 55 to 62 million sold packages in 2006 and 2009 respectively (INFARMED, 2006; 2009).

Table 1.5: Portugal in World medicine situation in 2000 (WHO, 2004)

Production and trade	**Million US$**
Production	2 459 635*
Importations	699 953
Exportations	171 707
Total sales	**Number**
Licensed brands	236 131
Original brands	679 797
Other brands	515 540
Patent brands	81 574
Unbranded	9 547
Expenditure	**US$**
Total expenditure on health (per capita at average exchange rate)	934
Total expenditure on pharmaceuticals (% total expenditure on health)	23.1
Total expenditure on pharmaceuticals (per capita at exchange rate)	216
Government expenditure on pharmaceuticals (per capita at exchange rate)	151
Private expenditure on pharmaceuticals (per capita at exchange rate)	65
Private expenditure as % total health spending	32.9*

* represents available 1997 data

Table 1.6: **Evolution of the top 50 APIs with highest number of packages in the Portuguese National Health Service from 2006 to 2009 (adapted from INFARMED, 2007; 2010)**

Ranking (2006)	API	Packages (2006)	Position	Ranking (2009)	API	Packages (2009)
1	**paracetamol/acetaminophen**	3 485 502	=	1	**paracetamol/acetaminophen**	3 642 302
2	**alprazolam**	2 777 192	↓	2	simvastatin	2 941 467
3	**diclofenac**	2 009 755	↓	3	metformin	2 429 287
4	trimetazidine	1 978 072	↓	4	alprazolam	2 280 686
5	simvastatin	1 961 484	↑	5	amoxicillin + clavulanic acid	2 239 124
6	nimesulide	1 929 370	↓	6	trimetazidine	2 102 559
7	amoxicillin + clavulanic acid	1 905 374	↑	7	omeprazole	2 029 495
8	metformin	1 829 256	↑	8	**ibuprofen**	2 016 009
9	lorazepam	1 774 078	=	9	lorazepam	1 909 169
10	indapamide	1 753 628	↓	10	**diclofenac**	1 817 520
11	omeprazole	1 535 483	↓	11	acetylsalicylic acid	1 778 370
12	**bromazepam**	1 427 739	↓	12	indapamide	1 743 762
13	**ibuprofen**	1 416 708	↑	13	nimesulide	1 510 441
14	**diazepam**	1 334 654	↓	14	gliclazide	1 355 017
15	gliclazide	1 261 323	↑	15	influenza vaccines	1 343 266
16	ethinylestradiol + gestodene	1 119 474	↓	16	furosemide	1 312 465
17	furosemide	1 044 148	↑	17	**bromazepam**	1 196 396
18	zolpidem	1 020 976	↓	18	flavonoids	1 170 030
19	flavonoids	1 005 970	↑	19	clopidogrel	1 153 693
20	influenza vaccines	993 044	↑	20	losartan + hydrochlorothiazide	1 139 107
21	amlodipine	967 452	↓	21	**diazepam**	1 130 220
22	acarbose	922 185	↓	22	bisoprolol	1 126 119
23	alendronic acid	854 164	↓	23	zolpidem	1 078 175
24	losartan + hydrochlorothiazide	835 205	↑	24	irbesartan + hydrochlorothiazide	1 021 011

Table 1.6: (Continuation).

25	atorvastatin	818 039	↓	25	levothyroxine sodium	963 390
26	bisoprolol	813 321	↑	26	tamsulosin	951 188
27	azithromycin	800 945	↓	27	perindopril	941 067
28	clopidogrel	799 096	↑	28	betahistine	934 658
29	irbesartan + hydrochlorothiazide	796 793	↑	29	rosuvastatin	913 081
30	nifedipine	777 566	↓	30	azithromycin	912 055
31	acetylsalicylic acid	774 849	↑	31	lansoprazole	856 467
32	perindopril	766 600	↑	32	pantoprazole	851 273
33	levothyroxine sodium	750 326	↑	33	valsartan + hydrochlorothiazide	831 899
34	glibenclamide	746 375	↓ 100	34	ethinylestradiol + gestodene	828 145
35	domperidone	723 191	↓	35	amlodipine	826 128
36	glucosamine	722 271	=	36	glucosamine	811 444
37	valsartan + hydrochlorothiazide	720 017	↑	37	domperidone	775 022
38	betahistine	695 494	↑	38	allopurinol	771 752
39	ciprofloxacin	681 745	↓	39	nifedipine	769 754
40	**fluoxetine**	675 111	↓	40	acarbose	744 087
41	tamsulosin	673 969	↑	41	**fluoxetine**	708 266
42	ambroxol	647 132	*	42	losartan	702 736
43	amoxicillin	633 260	↓ 55	43	atorvastatin	694 185
44	allopurinol	625 868	↑	44	alendronic acid	693 515
45	**salbutamol**	625 638	↓ 62	45	etoricoxib	680 552
46	lansoprazole	622 581	↑	46	desloratadine	669 753
47	citicoline	608 787	↓58	47	ciprofloxacin	640 648
48	sertraline	602 221	=	48	sertraline	630 785
49	acetylcysteine	601 254	*	49	ramipril	623 243
50	etoricoxib	583 958	↑	50	alendronic acid**	618 178

* out of the top-100 list; ** repetition of the same API; APIs in bold were selected and detected in Arade River, Portugal (see Chapter 2).

1.2 Pharmaceutical Products Usage

Common pharmaceutical dosage forms are: aerosols, capsules, creams, drops, emulsions, enema, gels, implants, inhalations, injections, irrigations, liniments, lotions, nebulizers, ointments, pastes, pills, powders, solutions, suppositories, suspensions, tablets, transdermal patches, etc. Besides the API, these compounds also include excipients, which comprise the product's delivery system, characterized for: a) transporting the API to the site in the body where the drug is intended to exert its action; b) restricting the drug from early release in the assimilation process; c) promoting the stability of the drug for maximum effectiveness at time of use; d) promoting the cleavage of the drug into small particles allowing a faster reach into the blood stream; or finally e) favoring the identification of a drug product. Pharmaceutical excipients are essential components of a modern drug product, even though technically "inactive" from a therapeutic sense (Weiner and Kotkoskie, 1999; International Pharmaceutical Excipients Council of the Americas, 2008).

Considering that the main goal in pharmaceutical development is to discover molecules that are resistant to metabolic degradation processes and still, persist to exert the desired effect, results that inevitably, a part of the API is excreted unchanged, withstanding the wastewater treatment processes, and finally entering in most aquatic ecosystems (Anderson *et al.*, 2004).

1.3 Pharmaceuticals and Personal Care Products (PPCPs) and the Environment

For many years, persistent organic pollutants (POPs) (such as synthetically manufactured organic chemicals and metals) were recognized as the most important contaminants with considerable potential hazards to environmental and human health (Bahe *et al.*, 2005).

Only in the last two decades, environment exposure to PPCPs (thought to be harmless) has been considered as a major concern. This misconception may be due to the fact that their development and application in healthcare has always been looked as one of the greatest benefits in modern society, improving substantially the health and lifestyle of individuals (Roberts and Bersuder, 2006). However, the exponential growth of industrial economies and human population demographics resulted in their ubiquity in

the environment. As Metcalfe *et al.* (2004a) states API's potential to enter the environment concerns the amount sold, its pharmacokinetic behavior (API's interaction in the body like its adsorption, distribution, metabolism and excretion) and degradation rates (chemical, microbial or photolytic) in the WWTP and in the environment itself.

An emergent contaminant express certain features as: 1) occurrences and effects at very low concentrations, 2) undefined effects of chronic low level exposures on ecosystem and human health, 3) being unsuccessfully removed by typical waste treatment processes, and/or 4) being subjected to environmental dispersion and transportation after treatment at WWTP systems (Wall, 2004). APIs meet all the emergent pollutant requisites and therefore must be issued in environmental managing (Daughton, 2003; Khetan and Collins, 2007). In addition, WHO (2010; 2011) alerts to the importance of the rational use of medicines addressing to the correct, proper and appropriate use referring to two important factors which can ultimately be linked to API's increased presence in the environment: "1) More than 50% of all medicines are prescribed, dispensed or sold inappropriately, and half of all patients fail to take medicines correctly and 2) the overuse, underuse or misuse of medicines harms people and wastes resources".

1.3.1 Drug Metabolism and Excretion

APIs are usually ingested or absorbed orally, transported via the portal circulation to the liver, subjected to hepatic metabolism followed by elimination as bile or via the kidneys (Li, 2001). APIs are partially metabolized (biotransformed) by Phase I or Phase II reactions in the body, before finally being excreted via urine and faeces (Halling-Sørensen *et al.*, 1997; Daughton and Ternes, 1999; Kümmerer, 2004) (Figure 1.1).

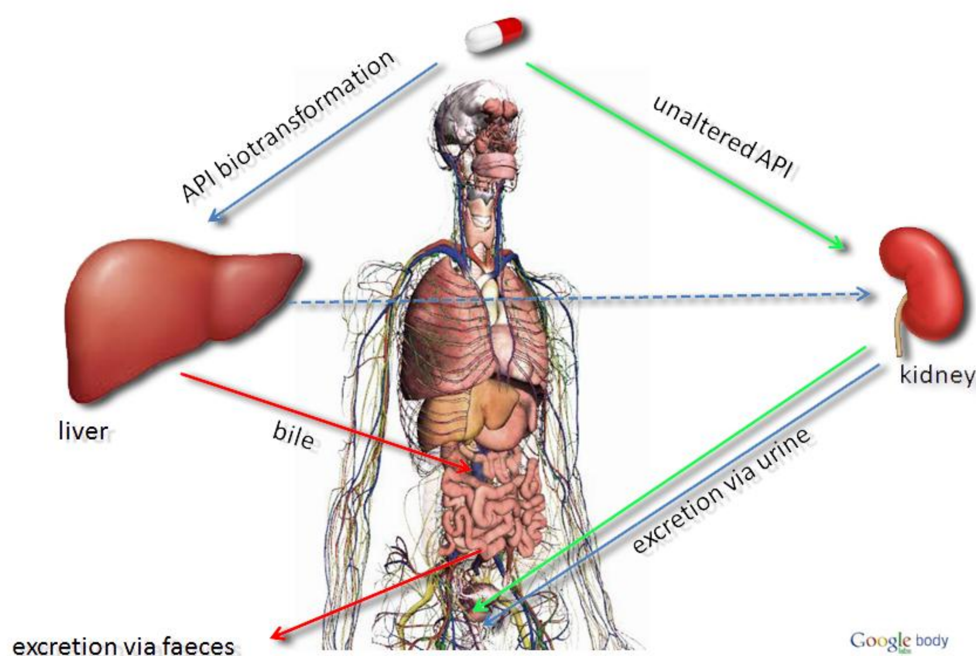

Figure 1.1: Scheme of API pathway in the body. Source of body figure: Google body

Elimination rates vary depending on the individual, drug and dosage (Bound and Voulvoulis, 2004; Bound *et al.*, 2006). Both Phase I and Phase II reactions change the physical chemical behavior of the parent substance. Phase I reactions typically consist of oxidation (which key enzymes comprehend CYP P450 family isoforms, like CYP3A4 responsible for 50% of known APIs xenobiotic metabolism) (Li, 2001), reduction or hydrolysis (for esters and epoxides), adding a reactive functional groups to the molecule and often converting it into a more toxic compound. Phase II or conjugation reactions include: acetylation, methylation, sulfation, glucuronide and glutathione conjugation, which promote the binding of functional groups to the compound. The key Phase II enzymes are UDP-dependent glucuronosyl transferase (UGT), phenol sulfotransferase (PST), estrogen sulfotransferase (EST) and glutathione-

S-transferase (GST) (Li, 2001). This metabolic strategy enhances excretion since it creates metabolites successively more polar and thereby more water-soluble than the parent compound (Halling-Sørensen *et al.*, 1997; Daughton and Ternes, 1999; Kümmerer, 2004; Josephy and Mannervik, 2006) (Figure 1.2).

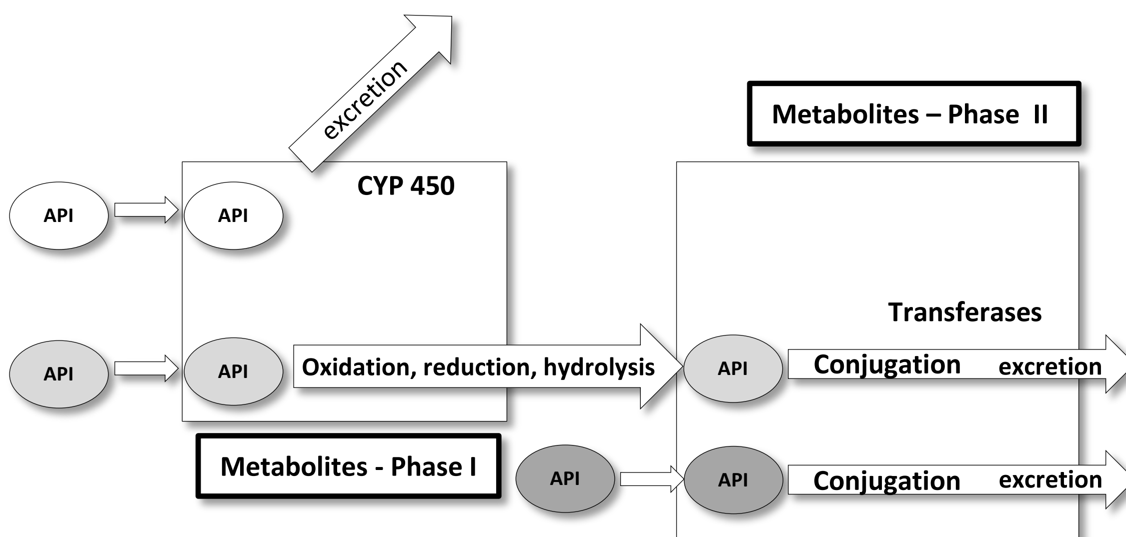

Figure 1.2: APIs metabolic biotransformation pathways to increase polarity and excretion (adapted from Daughton and Ternes, 1999)

APIs usage rely on its drug metabolism on different properties, namely 1) metabolic stability – a rapidly metabolized API implies multiple dosing to maintain therapeutic effect at plasma level; a slow metabolized API implies a slow elimination (prolonged half-life) and be unsafe to take; 2) drug-drug interactions – may occur due to the interference between each API metabolism when co-administrated and 3) drug toxicity - APIs may be transformed to non-toxic (detoxification) or more toxic (metabolic activation) by its metabolism (Li, 2001). Lastly, as Kümmerer (2004) refers an API parent compound may be excreted: unchanged, as a glucuronide or sulfate conjugate, as a metabolite or as a complex mixture of several metabolites.

1.3.2 APIs Sources and Routes in the Environment

The two major entering routes in the aquatic system are: (1) via domestic and hospital WWTP effluent discharges into receiving surface water and (2) via out-of-date or unwanted medicines disposal either flushed in the sink and/or toilet or as household waste which ends up in landfill sites leaking into surrounding water compartments (Figure 1.3). Other APIs pathways into the environment comprise: APIs applied in

aquacultures which are directly discharged in surrounding surface water; surface runoff after the application of biosolids/WWTP sludge and/or veterinary medicine treated animals manure as i.e. fertilizers; untreated sewage discharged into receiving waters (e.g., flood overload events); APIs application in food industry and disposal of APIs manufacturing wastes [usually low due to Good Manufacturing Practice regulations (GMPs)] (Christensen, 1998; Halling-Sørensen *et al.,* 1997; Ternes, 1998; Jones *et al.*, 2001; Heberer *et al.*, 2002; Andreozzi *et al.*, 2003; Metcalfe *et al.*, 2003a, b; Kümmerer, 2004; Bendz *et al.*, 2005; Castiglioni *et al*., 2005; Bound *et al.*, 2006; Lishman *et al.*, 2006; Bound and Voulvoulis, 2007; Gagné *et al*., 2007; Kümmerer, 2009; Santos *et al.*, 2010).

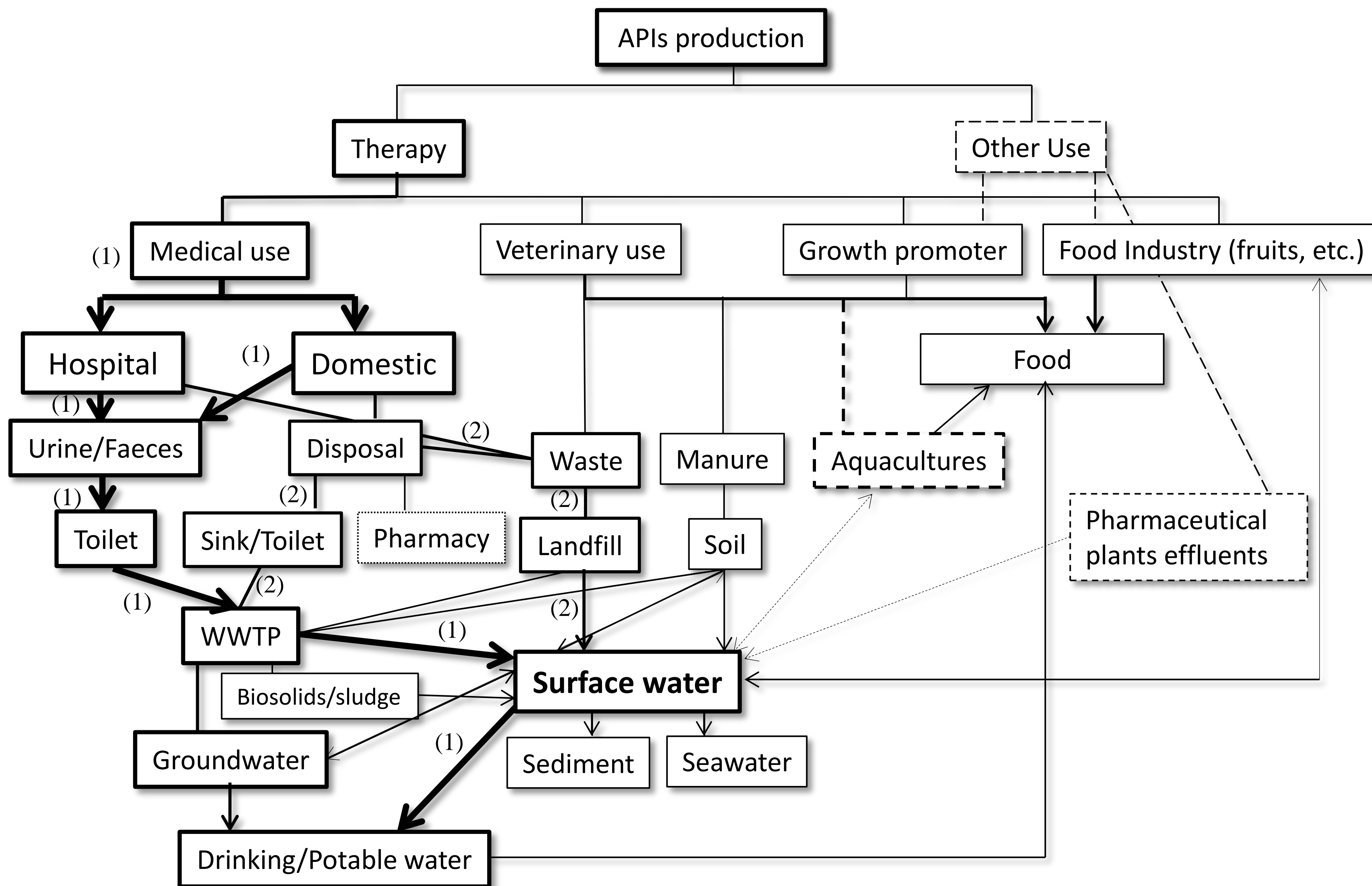

Figure 1.3: Pathways of APIs from production to the aquatic environment (adapted from Heberer, 2002; Kümmerer, 2004; Bound and Voulvoulis, 2007)

1.3.3 Fate of APIs in Waste Water Treatment Plants (WWTPs)

WWTPs are able to effectively control microbial pollution, eliminate organic matter and some nutrients (e.g., nitrogen and phosphorus). Generally the terms used to describe different degrees of treatment are:

- preliminary: include mostly coarse screening, grit removal and sometimes comminution/reduction of large objects;
- Primary: removal of organic and inorganic solids by sedimentation and the removal of materials that remain on surface by skimming - organic nitrogen, organic phosphorus, and metals associated with solids are also removed;
- Secondary: involves the removal of biodegradable dissolved and colloidal organic matter using aerobic biological treatment processes by aerobic microorganisms (principally bacteria) and apply several processes such as activated sludge, biofilters, oxidation ditches and rotating biological contactors (RBC);
- Tertiary and/or advanced wastewater treatment: is applied when further individual treatment processes are necessary to remove nitrogen, phosphorus, additional suspended solids, refractory organics, metals and dissolved solids. Advanced treatment processes are, in some cases, combined with primary or secondary treatment or used in place of secondary treatment;
- Disinfection: involves the injection of a chlorine solution and less frequently ozone and/or ultra violet (UV) irradiation (FAO, 1992).

These processes are nowadays usually based on complex biological treatment configurations by the combination of multiple reactors (anoxic, aerobic and anaerobic) and internal recycling between the different unit-processes (Rivas *et al.*, 2008). Yet, urban WWTPs continuously receive streams that include several different trace polluting compounds (either synthetic and/or natural), for which conventional treatment technologies have not been specifically designed, namely for active pharmaceutical ingredients removal (Carballa *et al.*, 2004; Reif *et al.*, 2008).

WWTPs play a critical role in the partitioning of APIs (largely depending on its degree of polarity and adsorption coefficients) into two exposure pathways associated with the liquid and the solid phase. Non-polar molecules and/or with high adsorption coefficient

(such as: musk and estrogens) are retained in sludge material, while the polar ones and/or with low adsorption coefficients (such as: anti-inflammatories and antibiotics) are likely to remain in the aqueous phase. The removal of APIs is affected by various factors such as: the chemical properties of the specific compounds, the age of the activated sludge, the dilution and temperature of the raw sewage, the treatment process employed, the environmental conditions (e.g. season), the characteristics of the influent, the hydraulic and solids retention time (HRT and SRT respectively) and the plant configuration (Kanda *et al.*, 2003; Carballa *et al.*, 2004; Clara *et al.*, 2004; Kreuzinger *et al.*, 2004; O'Brien and Dietrich, 2004; Clara *et al.*, 2005a, b; Vieno *et al.*, 2005; Tauxe-Wuersch *et al.*, 2005; Castiglioni *et al.*, 2006; Joss *et al.*, 2006; Zuccato *et al.*, 2006; Gros *et al.*, 2010).

The majority of the APIs are characterized as being: polar, non-volatile, low solubility and biodegradability. These characteristics favor their mobility into the receiving environment by escaping sedimentation and biological treatment in the WWTP (Carballa *et al.*, 2004; Bendz *et al.*, 2005). Additionally, these substances can bypass WWTPs through colloid-facilitated transport during periods of high effluent turbidity, overflow due to technical problems, floods, or high influent loads (Daughton and Ternes, 1999; Paxéus *et al.*, 2004). Although some APIs may have low persistence, the constant load may dominate over the transformation rate in the WWTPs (Bendz *et al.,* 2005).

Even though generally reported removal rates (RE%) data intervals vary for each individual API. Most studies refer that individually APIs elimination from municipal WWTPs is often incomplete (ranging on average between 50% and 99% of efficiency) which ultimately enables them to reach the environment at continuous influx (Christensen, 1998; Halling-Sørensen *et al.*, 1997; Ternes, 1998; Stumpf *et al.*, 1999; Heberer *et al.*, 2002; Andreozzi *et al.*, 2003; Metcalfe *et al.*, 2003b; Bendz *et al.*, 2005; Lindqvist *et al.*, 2005; Castiglioni *et al.*, 2006; De Lange *et al.*, 2006; Han *et al.*, 2006; Lishman *et al.*, 2006; Bound and Voulvoulis, 2007; Gagné *et al.*, 2007; Gros *et al.*, 2010). Some APIs can be easily removed during wastewater treatment, mainly by adsorption and biodegradation like analgesics acetylsalicylic acid (ASA), fenofibrate, and paracetamol/acetaminophen; some antibiotics and estrogens such as estrone (E1), 17-estradiol (E2), and 17-ethinylestradiol (EE2) also exhibit high removal rates when exposed to conventional activated sludge treatment. For instance, analgesic ibuprofen

exhibit high rates of oxic degradation, while iodinated X- ray contrast media remain unaffected by conventional activated sludge (Kümmerer, 2004).

Additionally, Gros *et al.* (2010) verified three different behaviors linking RE% with therapeutic class in WWTPs: (a) an increase in APIs concentration through its passage in the WWTP - macrolide antibiotics, anti-epileptic carbamazepine, benzodiazepines and serotonin reuptake inhibitors presented poor or no elimination at WWTP therefore exhibiting higher concentrations in wastewater effluents; (b) no significant to medium removal - lipid regulators, fluoroquinolone, tetracycline antibiotics, anti-cholesterol, antihistaminics, β- blockers, β-agonists and anti-diabetic glibenclamide were partially degraded with either average removal efficiencies between 40 and 60–70% or in another situations were not eliminated at all and (c) high removal efficiency - NSAIDs and the antihypertensive enalapril shown consistently high removal rates, except diclofenac (0 to 100%).

Although the APIs more frequently detected in receiving river waters generally match with those that are more ubiquitous in wastewater effluents, the highly consumed analgesics and anti-inflammatory drugs (like ibuprofen) are an exception since even after efficient removal by wastewater treatment this process is not enough to prevent their occurrence at considerable concentrations in river waters (Gros *et al.*, 2010).

1.3.4 APIs Transformation and Degradation in the Environmental

As Kümmerer (2009) refers an API can undergo different structural changes such as biotic (biotransformation, biodegradation) and non-biotic (oxidation, hydrolysis and photolysis) transformation processes after its introduction in different environmental compartments such as surface water, soil or sewage which alter their physico-chemical and pharmaceutical properties (as described in Figure 1.4).

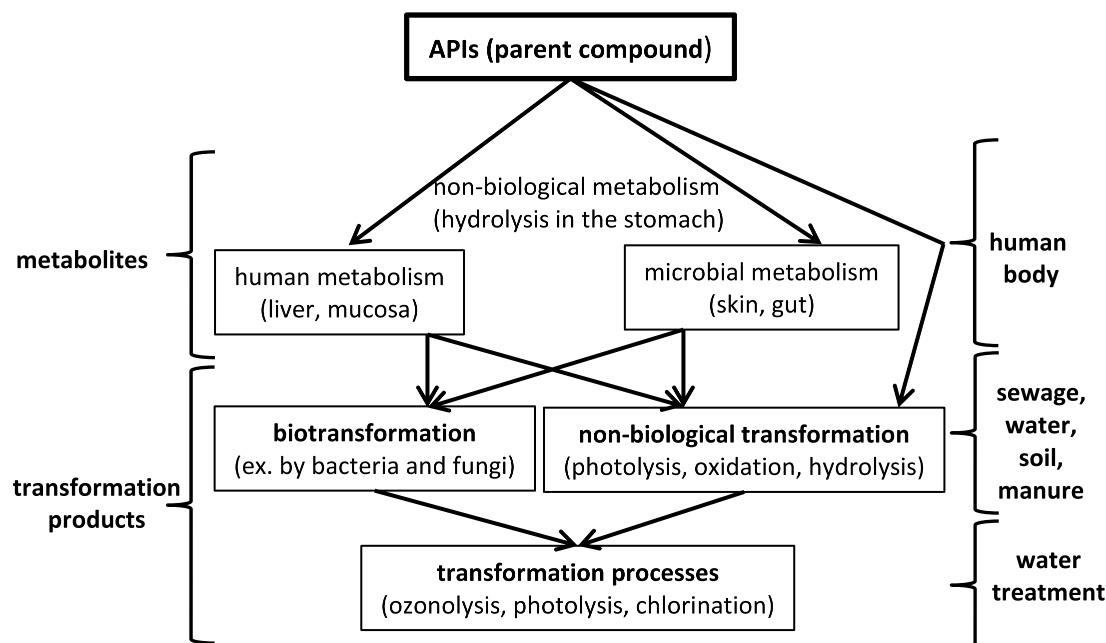

Figure 1.4: APIs metabolites and transformation products (adapted from Kümmerer, 2009)

The transformation and/or degradation of the parent compound when complete involve mineralization to low molecular weight compounds (i.e. carbon dioxide, sulfate, nitrate) or when the degradation is partial the process stops before mineralization and the intermediates are formed i.e. stable product of biotransformation, can be more stable than the parent compound itself and often have comparatively a higher accumulation potential (Kümmerer, 2009; Santos *et al.*, 2010). Bacteria and fungi are the organisms most capable to degrade organic compounds. Bacteria are particularly efficient in the aquatic environment biodegradation and fungi in soils biodegradation. For this reason, microbial degradation will be slower in surface water than in the sewage system due to its lower bacterial density and lower diversity (Kümmerer, 2009). Nevertheless, Sammartino et al. (2008) reports that APIs due to their specific action and stability are not subjected to microbial degradation, and on the contrary they can act reducing the bacterial flora present in the wasting site and therefore the non-biotic process of photodegradation can be considered the main way to eliminate APIs and their residues from the environment. Photodegradation comprises C-C bond break and in cases the break of the entire molecule by the action of the UV component of the solar radiation, this mechanism is dependent of e.g. UV radiation energy and intensity, latitude (seasonal and geographical variation), physical state of the APIs' molecule, chemical parameters such as pH, presence of photosensitizes (e.g. nitrates, humic acids) (Andrezotti *et al.*, 2003; Sammartino *et al.*, 2008; Santos *et al.*, 2010). Even if these

metabolic and transformation processes usually lead to decreases in APIs toxicity in the environment, the inverse can occur e.g. in the case of pro-drugs (Kümmerer, 2009). Finally, the inefficiency or insufficient action of the natural degradation processes is demonstrated by the presence of APIs in the aquatic system implicating potential toxicological and ecological problems (Sammartino *et al.*, 2008).

1.3.5 Occurrence in the Aquatic Environment

The first report featuring the occurrence of pharmaceuticals in the environment was published by Garrison *et al.* (1976). Pharmaceuticals are ubiquitous in the environment. More than 160 from all therapeutic classes have been screened at a concentration range from $ng.L^{-1}$ to $\mu g.L^{-1}$, in surface, groundwater, raw and treated wastewater, soil, biosolids, sediment samples and even drinking water worldwide including the Arctic environment (Kümmerer, 2009; Daughton *et al.*, 2010; Santos *et al.*, 2010). In order to trace APIs in water, concentration and extraction are required analytical procedures, allowing both the concentration of the analyte to measurable concentrations and the sample cleanup which removes analytical interferences. Solid phase extraction (SPE) is the method most commonly used for the extraction/concentration of all classes of APIs from aqueous samples using different types of sorbents (e.g. hydrophilic-lipophilic balanced (HLB), C18, polystyrene-divinylbenzene (PS-DVB) or mixed-mode cationic exchange (MCX) depending on the polarity of the selected compounds but also solid phase microextraction (SPME) and liquid-liquid extraction (LLE). These processes of extraction may be followed either by derivatisation and instrumental analysis: gas chromatography (GC), gas-chromatography-mass spectrometry (GC-MS) (usually for acidic pharmaceuticals quantification), high performance liquid chromatography (HPLC) or followed directly by liquid chromatography-mass spectrometry (LC-MS-MS) or liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS-MS) and ultra-performance liquid chromatography–quadrupole–time-of-flight mass spectrometry (LC-Q-TOF-MS) (Ternes, 2001; Weigel *et al.*, 2004a; Alvarez *et al.*, 2005; Petrovic *et al.*, 2006; 2007; Radjenovíc*et al.*, 2007; Togola and Budzinski, 2008; Gros *et al.*, 2010; Buchberger, 2011; Munaron *et al.*, 2012).

Additionally, passive samplers such as Polar Organic Integrative Sampler (POCIS) may be used to overcome the process of pre-concentration (Weigel *et al.*, 2004a; Togola and Budzinski, 2008; Buchberger, 2011) (see further detail in Chapter 2). The top detected

APIs therapeutic classes in the environment are described thoroughly by Santos *et al.* (2010) (Figure 1.5) in which NSAIDs are the class more recurrent whereas antipsychotics are the least present in the aquatic systems. The following Chapters 2, 3, 4 and 5 give a general idea of the maximum concentrations detected for some of these compounds in different aquatic matrices.

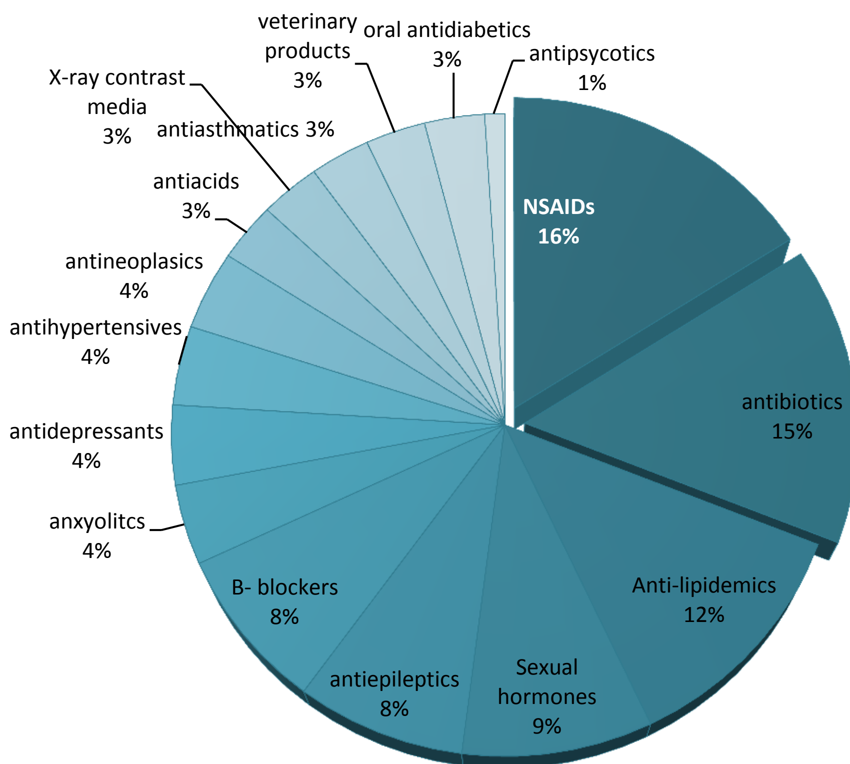

Figure 1.5: Therapeutic classes present in the environment between 1997 and 2009 (Santos *et al.*, 2010)

1.4 Current Legislation, Environmental Risk Assessment (ERA) of APIs

There is, since 1993 an acknowledgement of the relevant impact of APIs on the ecosystem by the European Medicine Evaluation Agency (EMEA) and U.S. Food and Drug Administration (FDA) since their current guidelines implicate that in order to authorize any novel API commercialization it is mandatory to perform an ERA. Broadly, an ERA is defined "as the procedure by which the likely or actual adverse effects of pollutant and other anthropogenic activities on ecosystems and their components are estimated with a known degree of certainty using scientific methodologies" (Depledge and Fossi, 1994). The ERA of APIs involves a two phase process: Phase I - is limited on the estimation of parental API or its metabolites environmental exposure, if the predicted environmental concentration (PEC) exceeds a threshold value that poses environmental concern additional studies on environment fate and effects are necessary in a second phase (Phase 2). In Europe that happens when the API's PEC exceeds 10 $ng.L^{-1}$ in surface waters whereas in the U.S. is 1000 $ng.L^{-1}$; Phase 2 - includes a hazard quotient approach, estimated by the ratio between PEC and predicted no effect concentration (PNEC) for various compartments that has to be < 1 (FDA, 1998; EMEA, 2006; 2007; EEA, 2010; Santos *et al.*, 2010; Buchberger, 2011).

A good portrait of this process complexity is the one applied to the French situation, this prioritization methodology based itself on the APIs threshold values of PECs established by the EMEA ranking APIs from class IA – "highest risk compound" to IV – "very low risk for the environment" (Besse and Garric, 2008) (see Phase 1: Figure 1.6 and Phase 2: Figure 1.7). Although there is the possibility to estimate the PEC for several APIs, it is very difficult to calculate PNEC for most APIs since their ecotoxicological data is still too scarce. Nonetheless as an example, NSAID diclofenac (DCF) (further characterized in Section 1.9.2. and Chapter 4) was recently the first API to be included in the new list of priority substances adopted by the European Union (European Commission, 2012a, b) and previously classified as a class IIA "potentially hazardous compound".

Still, based on the ERA process, an API applied in Human medicine may not be refused to be marketed, while a veterinary use one will be refused or need further risk mitigation measures (FDA, 1998; EMEA, 2006; 2007; EEA, 2010; Santos *et al.*, 2010; Buchberger, 2011). Finally, concerning known ERA on APIs results, EEA (2010) refers

that 95 % of the applied APIs were not readily biodegradable according to OECD Test 301 (OECD, 1992) and 15% of the human pharmaceuticals were persistent in water and 50% in the sediment according to and 308 (OECD, 2002)[1].

1.4.1 "Green Chemistry"

Following the same logical principles of "Green Chemistry" which intend to eliminate and/or minimize the usage and production of ecologically hazardous reagents and design of alternative synthesis pathways, "Green Pharmacy" involves, as proposed by Daughton (2003), the application of several strategies and measures to reduce the impact of APIs in the environment. For instance developments on: 1) drug design – i.e. increasing the specificity of API' mode of action (MoA) at the target receptor would greatly favor the evaluation of potential effects on non-target species; 2) drug delivery – i.e. provide prescriptions at lower doses and/or promote individualization of therapy; 3) packaging – i.e. reduce packages size, offer unit doses alternative and finally 4) disposal – providing consumer-oriented guidance on the package for how to dispose unused medicines). Furthermore, "Green" APIs would have the advantage of while enhancing and/or maintaining therapeutic efficacy improve APIs susceptibility to biodegradation, photolysis, or other physicochemical alterations to convert them to harmless end products (Daughton, 2003). Green and sustainable pharmacy is an emerging topic and is at early stages of implementation being currently not a high priority for the pharmaceutical industry (EEA, 2010). Most of all, since very little is yet known concerning environmental effects, fate and behavior of APIs it is crucial to create awareness and inform the general population, health care practitioners and manufacturers on these matters (Daughton, 2003; EEA, 2010).

[1] Note: OECD Test 301 is based on the determination of parameters in aquatic environments under aerobic conditions such as dissolved organic carbon (DOC), CO_2 production and oxygen uptake. The test is passed if in a 10 days window (within 28 day period) 70% removal of DOC and 60% of theoretical oxygen demand (ThOD) or carbon dioxide production ($ThCO_2$) (OECD, 1992); OECD Test 308 is based on the determination of aerobic and anaerobic transformation in aquatic sediment systems parameters (OECD, 2002)].

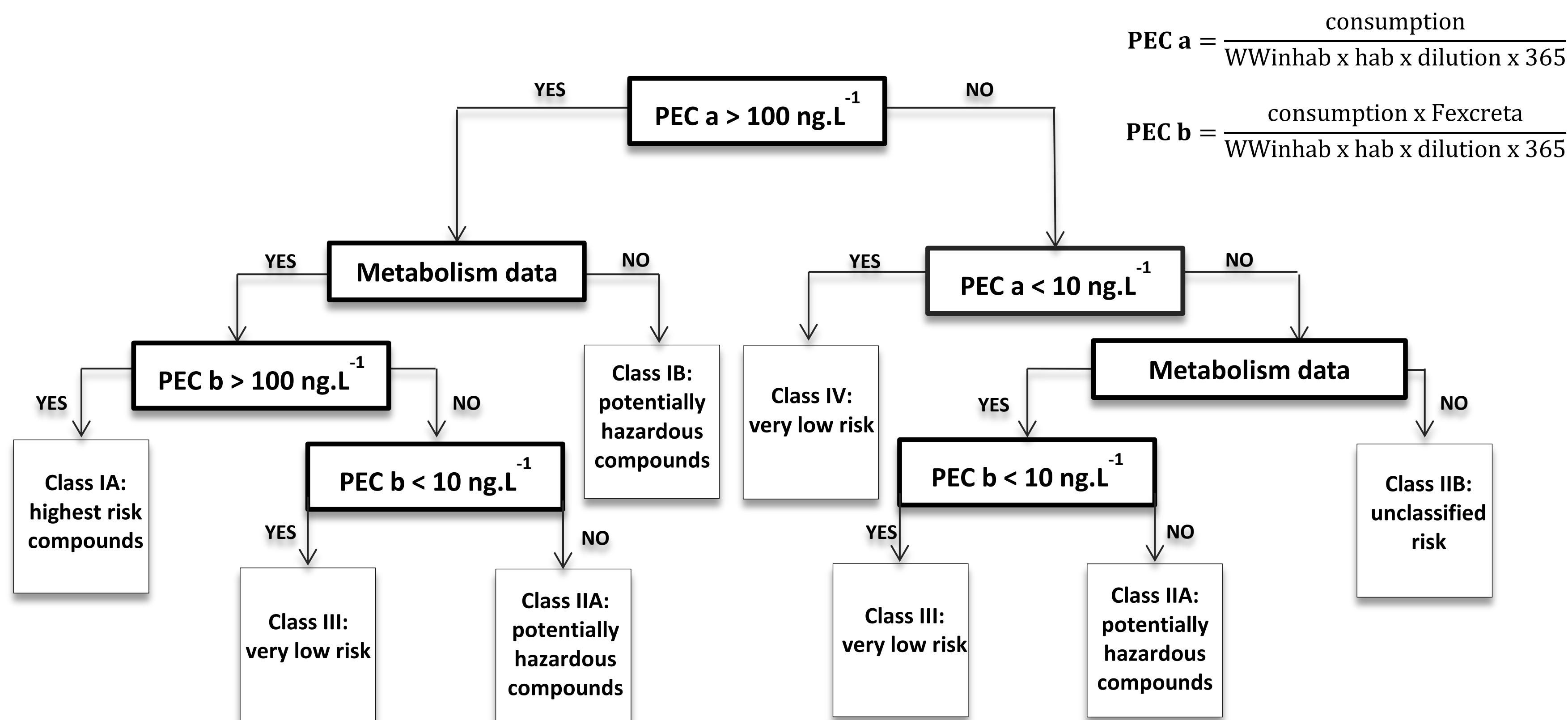

Figure 1.6: Phase I of the prioritization methodology proposal based on EMEA guidelines applied to French situation. PECa assumes the excretion of the API 100% as parental compound and 0% removal rate at WWTP; PECb takes in consideration the real amount of parental compound excreted (from Besse and Garric, 2008).

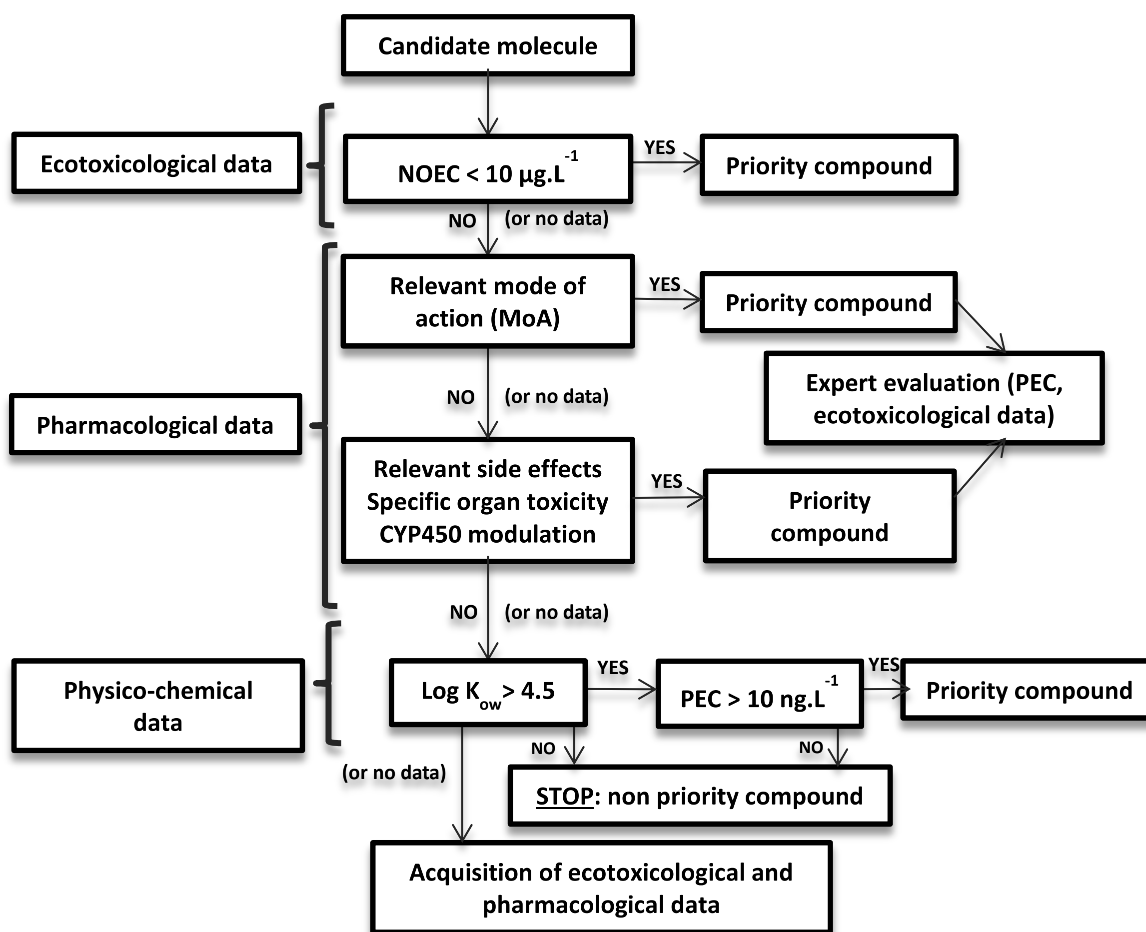

Figure 1.7: Phase 2 of the prioritization methodology proposal based on ecotoxicological, pharmacological and physico-chemical data. The YES is chosen only in the case of agreement with two criteria (adapted from Besse and Garric, 2008).

1.5 Ecotoxicological Effects on Aquatic Organisms

As mentioned previously, APIs are designed, for human and veterinary health practice, to exert a given therapeutic action targeting specific metabolic and molecular pathways (Fent *et al.*, 2006a, b). Even though APIs are claimed to be the most well-studied chemicals (Van der Ven *et al.*, 2006), their reactivity, MoA and associated side-effects are still not completely understood (Fent *et al.*, 2006a). Potentially when present in the aquatic ecosystem APIs will affect the same pathways in non-target organism with identical or similar target organ tissues, cells or biomolecules with analogous MoA (Fent *et al.*, 2006a). As referred by these authors, the MoA of NSAIDs (see Section 1.10.1) inhibit cyclooxygenase isoforms (COX-1 and -2) and consequently prostaglandins biosynthesis. Invertebrates also possess prostaglandins and COX activity (Osada and Nomura, 1990; Gagné, 2007; 2011). Another example is the antidepressant fluoxetine (see Section 1.10.2) action as a serotonin re-uptake inhibitor (SSRI) and serotonin (5-hydroxytrypamine or 5-HT) being also present in bivalves (although mediating endocrine functions) (Fong, 1998; Gagné and Blaise, 2003; Gagné *et al.,* 2007; 2011). In order to support an ERA several ecotoxicological studies have to be performed on organisms (i.e. algae, zooplankton, invertebrates and fish) such as acute (short-time exposure) and chronic (long-time exposure) toxicity bioassays related either to survival rate (lethal concentrations) or to other specific parameters (such as reproductive, feeding, growth alterations rates) occurring through the exposure to single or mixture to one or more concentrations of pharmaceutical compounds. For this matter, it is highly recommended the consultation of WikiPharma, EPA ECOTOX and European Commission Joint Research Center databases which enable easy and open access to up-to-date data related to most APIs acute and chronic toxicity effects on many different species (search should be made, either by name or CAS number). Although present knowledge on APIs effects in non-target organisms is growing at a good pace as seen by the evident increase of peer-reviewed publications on the matter (Fent *et al.*, 2006a; Kümmerer *et al.*, 2009; Santos *et al.*, 2010), very few studies have included the potential biochemical, metabolic and physiological response changes in aquatic organisms derived from chronic environmental realistic exposure concentrations to APIs using a multibiomarker analysis approach (see Section 1.6). As referred Van der Oost *et al.* (2003) the knowledge and understanding of the relationships between biomarker and bioassay responses is essential for a more reliable and holistic ERA.

1.5.1 Mixture Toxicity

Although individual APIs concentrations can often be considered low to cause any environmental risk, in an ecotoxicological point of view, a realistic scenario should consider that APIs are often (depending on the therapeutic intention) applied jointly and the overall input in the surface waters refers to a rather complex mixture. Preliminary data reports the co-occurrence of fourteen APIs from eight different therapeutic classes in the Arade and Guadiana Rivers (Portugal) (Gonzalez-Rey *et al.*, unpublished data). It is crucial to address this issue, because even if the components of a mixture are present below their individual ERA threshold concentrations, as a mixture they can eventually contribute to an increased toxicity through combined modes of action. In order to anticipate the effects of a chemical mixture often its calculation derives from known toxicities of the mixture of the individual components (Schnell *et al.*, 2009). Concentration addition (CA) and independent action (IA) are the two concepts available for formulating the null hypothesis of additivity, this happens assuming that chemicals act exerting their effects without diminishing or enhancing each other's toxicity (Cleuvers, 2003; 2004). In APIs studies, CA assumes that each mixture component possesses similar pharmacological MoAs and accordingly applicable for APIs targeting the same molecular site; IA conversely assumes that components have dissimilar MoAs and interaction at different target sites nevertheless reaching a common toxicological endpoint although through distinct mechanisms within an organism (Cleuvers, 2003; 2004; Schnell *et al.*, 2009). Phenomena of synergism or antagonism can also occur independently of a similar or dissimilar MoA (Cleuvers, 2003; 2004). Synergism and antagonism interactions are defined in relation to this additivity assumption as upwards (i.e. higher than expected mixture toxicities) or downwards deviations (i.e. lower than expected mixture toxicities) (Cleuvers, 2003; Maria and Bebianno, 2011). Very few studies have addressed APIs mixture effects in non-target organisms.

1.6 Biomarkers

Biomarkers have been successfully applied to test xenobiotic compounds effects particularly environmental contaminants such as: metals, polycyclic aromatic hydrocarbons (PAHs), pesticides, endocrine disruptors, etc. Essentially, the majority of the biomarkers refers to a quantitative biologic response (either at molecular, histological, immunological, physiological or behavioral levels) induced in a sentinel

species, like mussels (see Section 1.8) by the presence or exposure to contaminants (Cajaraville *et al.,* 2000; Van der Oost *et al.*, 2003). Furthermore biomarkers, particularly molecular ones, are defined as "short-term indicators of long-term biological effects" as they can be used on the early warning signaling of a contaminant derived deviation (comparatively to an unstressed situation) ultimately allowing bioremediation strategies to prevent an irreversible damage on the ecosystem (Cajaraville *et al.*, 2000; Van der Oost *et al.*, 2003). There are three classes of biomarkers: 1) biomarkers of susceptibility: indicating the organism's inherent or acquired ability to respond to exposure to a specific xenobiotic substance (Van der Oost *et al.*, 2003); 2) biomarkers of effect: measurable biochemical, physiological or other alterations within organism's, for instance tissues, implying deleterious effect and 3) biomarkers of exposure: addressing the specific contaminant, its metabolite or product of interaction detection and quantification within the organism compartments (ex. tissues, cells). Although biomarkers indicative of exposure to contaminants are useful, biomarker of effects are more ecologically relevant (Cajaraville *et al.*, 2000).

1.7 Oxidative Stress

The occurrence of oxidative stress in aquatic organisms has been frequently associated with both natural abiotic factors and the presence of contaminants (Winston and Di Giulio, 1991; Winston *et al.*, 1996). Oxidative stress reflects an imbalance between the production and removal or scavenging of oxyradials or reactive oxygen species (ROS) [such as superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$)] (Figure 1.8). The latter is a potent oxidant which in elevated concentrations can be highly toxic to cell compartments (Winston and Di Giulio, 1991; Winston *et al.*, 1996). Several contaminants have the ability to promote ROS, especially the ones that undergo univalent metabolism to form redox-cycling intermediates (like PAHs and metals) (Winston and Di Giulio, 1991; 1996; Valavanidis, 2006) this is also the case of APIs since "their specific redox reactivity is the basis for their biological (therapeutic) effects, metabolism, elimination and toxicity" (Martín-Díaz *et al.*, 2009). Antioxidant systems are efficient protective mechanisms against ROS toxic action intercepting and inactivating their toxic action maintaining them at minimal intracellular levels. Therefore, due to their high sensitivity antioxidant systems assume an import role in environmental studies (Winston and Di Giulio, 1991; Winston *et al.*, 1996:

Cajaraville *et al.*, 2000; Livingstone, 2001; Regoli *et al.*, 2002a, b; Valavanidis *et al.*, 2006) and may be non-enzymatic (through the action of ascorbic acid and β-carotene) or enzymatic, through the action of enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathiones peroxidase (GPx) which synthesis adjusts and adapts to stress levels fluctuations (Fridovich, 1998; Valavanidis *et al.*, 2006). The assessment of these enzymes activities alterations is consequently considered an efficient oxidative stress biomarker (Cajaraville *et al.*, 2000; Regoli *et al.*, 2002a, b; Valavanidis *et al.*, 2006; Martín-Díaz, 2009; Maria and Bebianno, 2011).

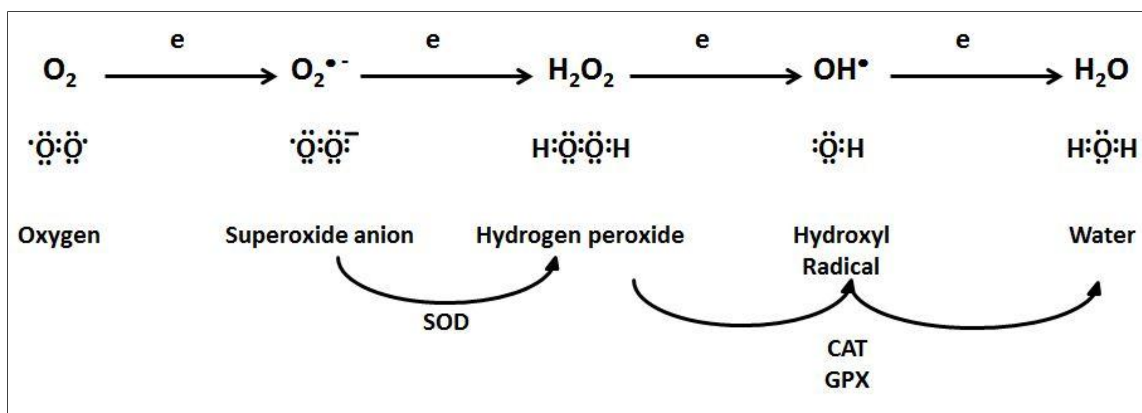

Figure 1.8: Diagram of reactive oxygen (ROS) formation. Adapted from Racila and Bickenbach (2009).

1.7.1 Antioxidant Enzymes: Oxidative stress biomarkers

SOD (EC 1.15.1.1) activity is one of the first defense mechanisms against oxidative stress, mostly acting on the catalytic conversion (dismutation) of the highly reactive $O_2^{\bullet-}$ anion into the more stable and less reactive H_2O_2. From then, H_2O_2 can easily cross the cellular membranes to be breakdown into H_2O and O_2 in the cytosol by CAT and glutathione peroxidases (Fridovich, 1998). CAT (EC 1.11.1.6) is located in the peroxisomes occurring in almost all aerobic organisms. This enzyme can also act as peroxidase, where organic substances like ethanol may act as a hydrogen donor (Santovito *et al.*, 2005). Glutathione is a water-soluble tripeptide constituted by the glutamine, cysteine and glycine amino acids present in abundance in most cells being both an important substrate for glutathione peroxidases and glutathione transferases but also as a ROS scavenger (Regoli and Principato, 2005; Fridovich, 1998; Townsend *et al.*, 2003). Glutathione can exist intracellularly either as GSSG or GSH (oxidized or reduced form respectively) and its antioxidant ability is associated to its thiol group (as a reducing agent). Therefore, GSH plays a significant role in electrophilic compounds

and peroxides detoxification via catalysis by glutathione S-transferases (GST) and glutathione peroxidases (GPx). Low GSH levels are associated to a higher sensitivity to prooxidants and increase of cellular membrane damage (Townsend *et al.*, 2003).

Glutathione reductase (GR) (EC 1.6.4.2) is a flavoenzyme, which regenerates GSH from GSSG and plays a crucial role in GSH turnover and in cellular antioxidant protection maintaining GSH in high concentrations and GSSG at low concentrations (Bashir *et al.*, 1995). As mentioned by Cossu *et al.*, (1997) decreased in GR activity may lead to GSH depletion if not corrected by new glutathione molecules synthesis. On another hand, Phase II glutathione-S-transferase (GST) (EC 2.5.1.18) has an important function as catalyst in the conjugation reaction with tripeptide glutathione converting xenobiotic molecules into more hydrophilic conjugates (more polar) therefore enhancing their excretion and detoxification from cells. In addition, GST is also a catalyst of selenium-independent glutathione peroxidase activity possessing also a protective role against ROS deleterious effects (Manduzio *et al.*, 2004).

Despite the collective action of these enzymes there is always a percentage of ROS that can overcome oxidative stress detoxification mechanisms, namely the highly toxic $OH^{\bullet}$ radical having an important oxidative potential with indiscriminate reactivity in cellular components that can cause severe alterations on several enzymatic activities and ultimately damage on biological membranes (lipid peroxidation - LPO) and DNA (Valavanidis *et al.*, 2006) (Figure 1.9).

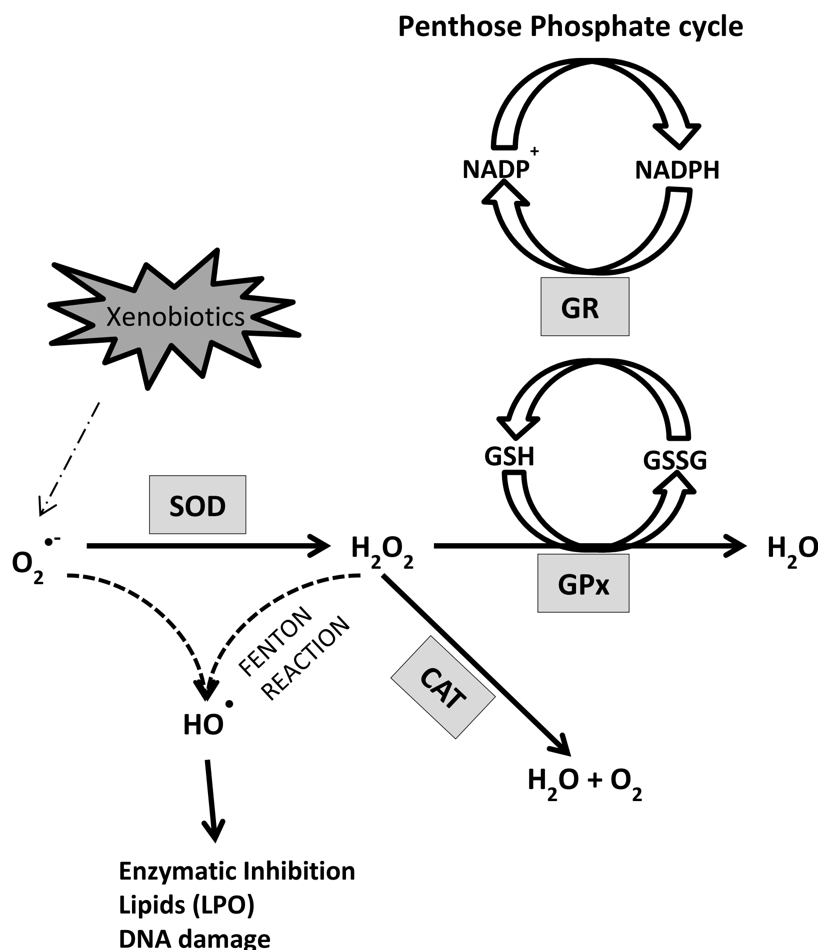

Figure 1.9: Antioxidant enzymatic reactions cascade. Adapted from Winston and Di Giulio (1991) and from Sigma-Aldrich official site (http://www.sigmaaldrich.com/technical-documents/articles/biofiles/cellular-derived-blood.html)

1.7.2 Lipid Peroxidation (LPO)

LPO is characterized by phospholipid membrane permeability loss, rigidity onset and consequently as an assault to cell membrane integrity (Valavanidis *et al.*, 2006). This phenomenon happen as a cascade of oxidative reactions set particularly in phospholipid membranes' polyunsaturated lipids (LH) whose high sensitivity to ROS action is due to their double bond structure. The final product of these reactions is the lipid hydroperoxide (LOOH) which can be decomposed into several reactive species, including lipid alkoxyl radicals, aldehydes (e.g. malondialdehyde, HOC–CH-CHO), alkanes, lipid epoxides and alcohols (Valavanidis *et al.*, 2006). The quantification of LPO enables the assessment of cell membrane damage, since it can induce deleterious effects in tissues and cells, and *in extremis* in DNA damage. Therefore, LPO is considered an efficient biomarker of damage cause by oxidative stress and extensively

reported in mussels (Rilkans and Hornbrook, 1997; Girotti, 1998; Valavanidis *et al.*, 2006; Gagné *et al.*, 2011).

1.8 Neurotoxic Effects

1.8.1 Acetylcholinesterase (AChE)

AChE activity has a critical role in cholinergic nervous function (ex. for muscular contraction and heart beating in bivalves) as the promoter of neurotransmitter acetylcholine (ACh) hydrolysis to choline and acetic acid (Tsuchiya *et al.*, 2004). AChE inhibition is considered as a biomarker of exposure to several contaminants such as organophosphorus pesticides, detergents and APIs (Ricciardi *et al.*, 2006; Matozzo *et al.*, 2005; Solé *et al.*, 2010) and has been successfully applied in *M. galloprovincialis* to assess neurotoxic effect (Bodin *et al.*, 2004; Banni *et al.*, 2010; Dondero *et al.*, 2010; Gorbi *et al.*, 2011).

1.9 Endocrine Disruption

One of the very first effects associated to exposure to SSRIs in aquatic organisms was the induction and potentiation of parturition/spawning (Fong, 1998; Fong and Molnar, 2008). Therefore, some APIs do have the ability to induce evidences of endocrine disruption in non-target organism. Endocrine disruption is often associated to a very diverse group of compounds including natural (17-estradiol - E2) and synthetic hormones (17α ethynylestradiol - EE2), polychlorinated biphenyls, organochlorine pesticides, plasticizers (like bisphenol A - BPA) and detergents (like nonylphenols - NPs). Many known endocrine disruptor compounds (EDCs) are estrogenic, affecting in particular reproductive functions. Additionally, due to its persistent nature and lipophilicity most xenoestrogens (and their metabolites) are bioaccumulated and biomagnified in environmental compartments, including marine biota, nevertheless most data concerning biological effects and mechanisms of action of EDCs on marine organisms derive from studies made on vertebrates and data on invertebrates are evidently lacking mainly due to the lack of knowledge of the invertebrates endocrine system (Porte *et al.*, 2006; Gagnaire *et al.*, 2009).

Vitellogenins (Vtg) precede egg-yolk protein vitellin (Vn) in oviparous species being naturally synthesized in females and inactive in males (Matozzo and Marin, 2008; Matozzo *et al.*, 2008). Whenever elevated levels are detected in males it is a sign of the

occurrence of endocrine disruption (ED) (Blaise *et al.*, 1999; Gagné *et al.*, 2002; Matozzo and Marin, 2008; Matozzo *et al.*, 2008). The measurement of alkali-labile phosphates (ALP) released by Vg after alkali hydrolysis is considered an effective and cost-effective method of indirectly assess xenoestrogenicity in bivalves and therefore ED biomarker in invertebrates (Blaise *et al.*, 1999; Gagné *et al.*, 2001; 2002; Porte *et al.*, 2006; Ortiz-Zarragoitia and Cajaraville, 2006; Matozzo and Marin, 2008; Matozzo *et al.*, 2008).

1.10 Selected Species

Mussels are economically valuable species, easy to cultivate or collect in coastal areas (Fuentes *et al.*, 2009). The Genus *Mytilus* has been successfully applied as an environmental sentinel species in numerous ecotoxicological studies (like "Mussel Watch" Program) (Goldberg *et al.*, 1978) due to its natural ability to accumulate contaminants from the surrounding water, wide distribution and long-life span (Cajaraville *et al.*, 2000; Porte *et al.*, 2006; Ricciardi *et al.*, 2006; Gorbi *et al.*, 2008; Cravo *et al.*, 2009; Dondero *et al.*, 2010; Ericson *et al.*, 2010; Ortiz-Zarragoitia and Cajaraville, 2010; Sureda *et al.*, 2010; Franzellitti *et al.*, 2011; Gonzalez-Rey *et al.*, 2011; Maria and Bebianno, 2011).

1.10.1 *Mytilus galloprovincialis* (Lamark, 1819) (Figure 1.8)

M. galloprovincialis have a wide native range distribution being present in the Atlantic Ocean coast (from the shores of the Irish Sea, where it co-occurs and hybridizes with *Mytilus edulis* Linnaeus, to Southern Spain) and in Western Mediterranean, Tyrrhenian, Adriatic, Aegean and Black Seas (Daguin and Borsa, 1999; Branch and Stefani, 2004).

Figure 1.10: Mussel *Mytilus galloprovincialis*. Source: http://eol.org/pages/449961/overview

This species has been introduced intentionally and/or accidentally via aquaculture, ballast waters or attachment to ships' hulls, etc. in South Africa, Asia (Hong Kong, Japan and Korea), Southeast Australia and West Coast of North America (Branch and Stefani, 2004). This mussel lives attached by segregated byssus threads on hard substrates (rocks and piers) within sheltered harbors, estuaries and open coast

rocky shores over the intertidal region to 40 m depth (FAO, 2012). It is a filter-feeder species and its diet consists of phytoplankton and detritus present in the surrounding water. Lastly, *M. galloprovincialis* possess gonochoristic reproduction (males and females spawn simultaneously) and its high fecundity is controlled by water temperature and food availability (Bayne, 1976; FAO, 2012).

1.11 Selected APIs for Exposure

The APIs selected in the present thesis (ibuprofen, fluoxetine and diclofenac) were chosen since they are amongst the top selling pharmaceuticals worldwide being at the same time the most frequently detected in the aquatic environment. Its general chemical properties are in Table 1.7. This section describes each API major characteristics, such as therapeutic action, pathways in the human body, metabolism and fate in the environment.

Table 1.7: Chemical properties of selected pharmaceuticals: IBU, DCF and FLX (adapted from Tixier *et al.*, 2003 and retrieved online: DrugBank; ChemID plus Advanced)

Therapeutic class	NSAIDs		SSRI
Properties	**IBUPROFEN (IBU)**	**DICLOFENAC Sodium (DCF)**	**FLUOXETINE Hydrochloride (FLX)**
CAS number	15687-27-1	15307-86-5	59333-67-4*
Molecular formula	$C_{13}H_{18}O_2$	$C_{14}H_{10}Cl_2NNaO_2$	$C_{17}H_{19}ClF_3NO$
Molecular weight	206.29	318.13	345
Water solubility	0.049 $mg.ml^{-1}$	2.43 $mg.ml^{-1}$	14 $mg.ml^{-1}$
pKa	4.91	13.4	10.06 (without HCl)
Henry's law constant	1.5×10^{-7}	4.75×10^{-12}	8.90×10^{-8}
Log K_{ow}	4.13-4.91	0.7	4.05

* (R-(-) fluoxetine hydrochloride) CAS number: 114247-09-5

1.11.1 Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

Antipyretic analgesics are a group of heterogeneous substances including acidic (like, NSAIDs) and nonacidic (like, paracetamol) drugs. NSAIDs are the most present therapeutic group in the aquatic system (Parolini *et al.*, 2009; Santos *et al.*, 2010) comprising top-selling non-prescription APIs such as: ibuprofen, naproxen and diclofenac. In the 70's, it was shown that the anti-inflammatory action of NSAIDs rests in their ability to promote the non-selective inhibition of cyclooxygenase (COX)-1 and -2 isoforms which mediate the biosynthesis of proinflammatory eicosanoid prostaglandins (PGs) from the phospholipid arachidonic acid (AA) (Vane *et al.*, 1998;

Fent *et al.*, 2006a; Hinz and Brune, 2006; Praveen Rao and Knaus, 2008). PGs produced during inflammatory states may significantly increase the excitability of nociceptive ("pain-receptor") nerve fibers contributing to the development of burning pain and therefore the action of NSAIDs arises from this peripheral sensitization prevention, diminishing pain perception (Hinz and Brune, 2006; DrugBank, 2012). Eicosanoids derived from AA three pathways metabolization (i.e. PG and thromboxane – from COX pathway; leukotrienes and lipoxins - from lipoxygenase (LOX) pathway and epoxyeicosatrienoic acids – from CYP P450 epoxygenase pathway) are involved in the regulation of inflammation, ion transport and neural and reproductive function (Heckmann *et al.*, 2007; 2008). The metabolization pathway of NSAIDs in humans and mammals is represented in Figure 1.11. In invertebrates, like molluscs, PGs act as "local" hormones critical in physiological functions such as: reproduction (gonadal development, spawning - behavior and oocyte fertilization/production control), water transport, osmoregulation ion balance, control of cardiac function (in *Octopus vulgaris*) and chemical defense (Ruggeri and Thoroughgood, 1985; Osada and Nomura, 1990; Rowley *et al.*, 2005).

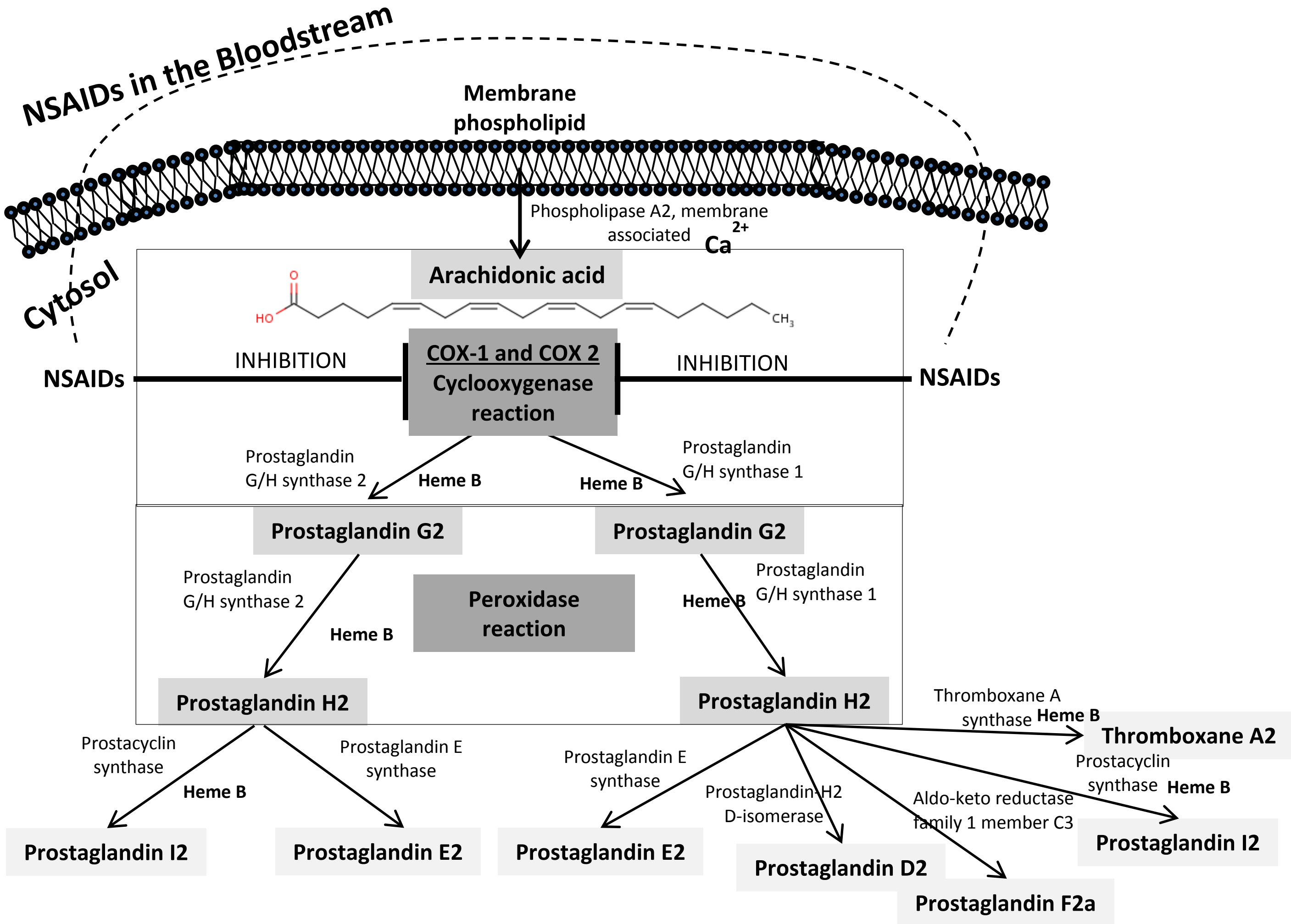

Figure 1.11: NSAID pathway in humans. Adapted from Hinz and Brune, 2006 and DrugBank (http://www.drugbank.ca/drugs/DB00586)

1.11.1.1 Ibuprofen (IBU)

IBUPROFEN (IBU), (2-(4-isobutylphenyl) propanoic acid (Figure 1.12), is known as prototypical NSAID with analgesic and antipyretic properties used in the treatment of fever and pain. Is commercialized as a non-prescription drug and most known brand names are Advil® and Brufen® (Zwiener *et al.*, 2002; DrugBank, 2012). After application IBU is rapidly metabolized and excreted in the urine, approximately 15% as the parent compound and in form of its metabolites: hydroxyibuprofen (OH-IBU), carboxyibuprofen (CA-IBU) and carboxyhydratropic acid (CA-HA) (Ternes, 1998; Zwiener *et al.*, 2002). At WWTPs IBU shown removal rates averaging 91% (Tixier *et al.*, 2003; Khetan and Collins, 2007; Quintana *et al.*, 2005; Palmer *et al.*, 2008; Gros *et al.*, 2010). Direct phototransformation can be neglected because this compound does not absorb sunlight, though its elimination by sedimentation is relevant due to a high sorption coefficient to particles combined with particulate organic carbon (Tixier *et al.*, 2003). IBU is defined as a class IA compound which defines it as posing a "high environmental risk" (Besse and Garric, 2008).

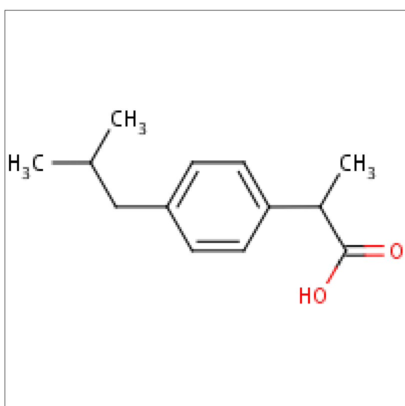

Figure 1.12: IBU chemical structure. Source: ChemID plus Advanced.

1.11.1.2 Diclofenac (Sodium Salt)

Diclofenac sodium salt (DCF) or 2-[(2,6-dichlorophenyl)amino]benzeneacetic acid sodium salt (Figure 1.13) is the most highly consumed anti-inflammatory predominantly prescribed for the treatment of rheumatic (arthritic) pain. Its administration is usually oral or topical and common brand names are Voltaren® and Cataflan®. DCF appears to be less active on COX-1 as compared to COX-2 (Hinz and Brune, 2006). DCF undergoes a considerable first-pass metabolism that causes its limited oral bioavailability (about 50%), the lack of therapeutic effect imply an adaptation of the dosage or change of the drug (Hinz and Brune, 2006). The DCF metabolism in humans and animals has been studied extensively, DCF undergoes bioactivation by cytochrome P450 oxidation to hydroxylated derivatives including 4-hydroxy (OH) and 5-OH metabolites and minor metabolites include 3-OH, 3-OH-4-methoxy and 4,5-dihydroxy diclofenac (Marco-Urrea *et al.*, 2010). DCF is eliminated through metabolism and subsequent urinary and biliary excretion (DrugBank, 2012). Nevertheless, parental DCF is commonly found in water following a still contradictory removal rate at the WWTPs (averaging 58%) (Gros *et al.*, 2010; Marco-Urrea *et al.*, 2010). Direct phototransformation (by sunlight) is the main elimination process in surface waters nevertheless this process strongly time and site specific (Tixier *et al.*, 2003; Bartels and Tümpling, 2007). In a ecotoxicology perspective, DCF was one of the first APIs raising “a red flag” on APIs potential as an environmental contaminant when it was directly associated as the cause of Indian vulture (*Gyps* sp.) population decline after scavenging DCF-treated livestock carcasses (Oaks *et al.,* 2004). As mentioned previously according to Besse and Garric (2008) DCF is classified as a class IIA “potentially hazardous compound” and has been recently included in the list of priority substances in the field of water policy by the European Union (European Commission, 2012a, b).

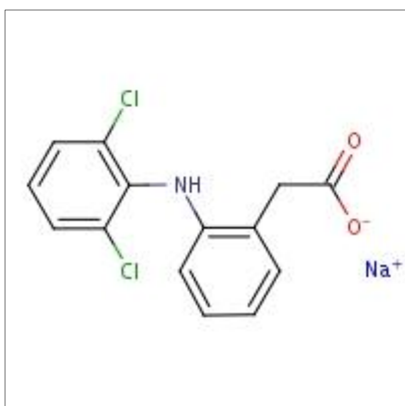

Figure 1.13: DCF chemical structure. Source: ChemID plus Advanced

1.11.2 Selective Serotonin Reuptake Inhibitors (SSRIs)

Selective serotonin reuptake inhibitors (SSRIs) have emerged as a major therapeutic advance in neuropsychopharmacology establishing the pathophysiological role of serotonin (5-HT) and confirming that neurotransmitter reuptake inhibition is an important therapeutic principle in affective and anxiety disorders treatment (Vaswani *et al.*, 2003). SSRIs were developed to inhibit/block 5-HT neuronal uptake pump, without affecting the various other neuroreceptors (i.e., histamine, acetylcholine and adrenergic receptors) or fast sodium channels like tricyclic antidepressants (TCAs), therefore avoiding its associated safety problems (Vaswani *et al.*, 2003). In humans, 5-HT modulates homeostasis between dopamine, noradrenaline and γ-aminobutyric acid (GABA) when this homeostasis gets disturbed, the onset of depression and anxiety occurs. Patients may respond differently to SSRI however that does not imply that one is more effective than another (Vaswani *et al.*, 2003). First SSRI antidepressant marketed was zimelidine which was banned due to serious cases of central and/or peripheral neuropathy, namely suicide tendency. Nowadays common SSRIs are fluoxetine, fluvoxamine, citalopram, paroxetine and sertraline (Vaswani *et al.*, 2003; DrugBank, 2012).

1.11.2.1 Fluoxetine

Fluoxetine (FLX) [(R-(-) fluoxetine hydrochloride)] or methyl({3-phenyl-3-[4-(trifluoromethyl)phenoxy]propyl})amine (Figure 1.14) is the most widely prescribed psychoactive agent in the market. Most common brand name is Prozac® and it is used to treat depression, bulimia, premenstrual dysphoric disorder, panic disorder and post-traumatic stress (Hiemke and Hartler, 2000; Kwon and Armbrust, 2006; DrugBank, 2012). After oral administration, FLX is almost entirely absorbed, undergoing extensive metabolic conversion to its active metabolite norfluoxetine in the liver. FLX is mainly excreted in urine, with less than 10% excreted unchanged or as fluoxetine N-glucuronide (Hiemke and Hartler, 2000; Vaswani *et al.*, 2003). FLX has a half-life of 1 - 4 days, whereas the half-life of norfluoxetine ranges between 7 and 15 days (Hiemke and Hartler, 2000). The pathway of FLX in humans and mammals is represented in Figure 1.15. Several studies reveal the occurrence of FLX in the environment, after a 60% removal rate at WWTPs. Photodegradation has a low contribution of to the dissipation of FLX in the aquatic systems (Kwon and Armbrust, 2006). According to Besse and Garric (2008), FLX is classified as a class III “very low risk” compound, nevertheless several ecotoxicological effects including bioaccumulation in several fish muscles (Brooks *et al.*, 2005) and induction of spawning in bivalves (Fong and Molnar, 2008; Lazzara *et al.* 2012; Bringolf *et al.*, 2010) have been attributed to FLX exposure.

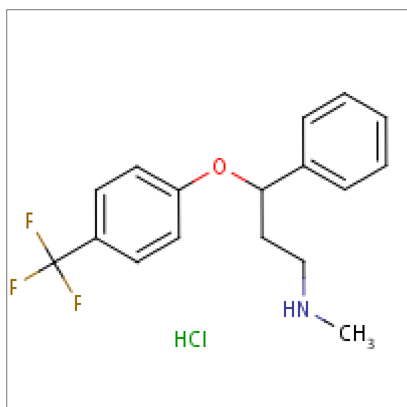

Figure 1.14: FLX chemical structure. Source: ChemID plus Advanced

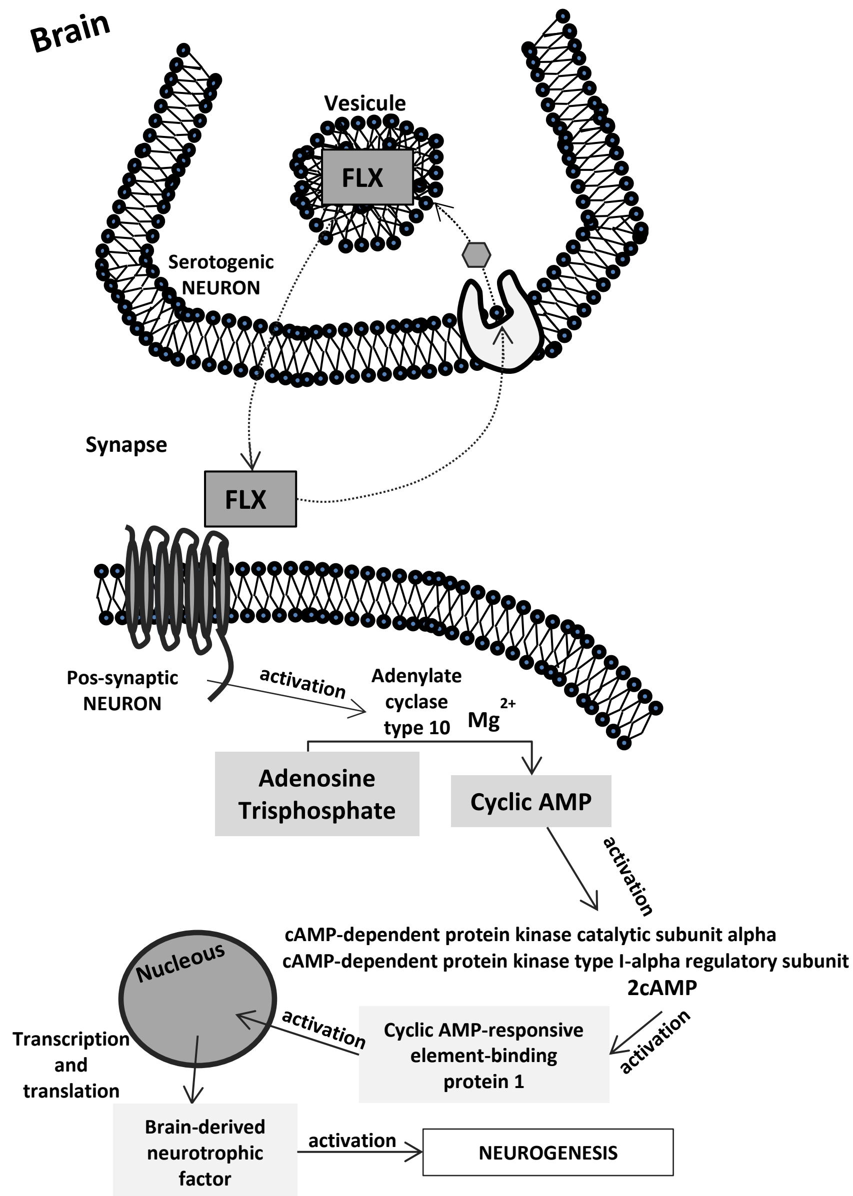

Figure 1.15: Fluoxetine pathway in human and mammals. Adapted from DrugBank: www.drugbank.ca/drugs/DB00472

1.12 Copper (Cu)

Briefly, copper (Cu) is considered one of the most important risk factors in the marine environment and a “classic” contaminant entering the aquatic environment through several pathways, including runoff resulting from mining activities, industrial activities, leaching from wood preservatives, and multi-application as antifouling agents in paints, fertilizers (Bonnard *et al.*, 2010; Maria and Bebianno, 2011) and lately as copper oxide nanoparticles containing products (Gomes *et al.*, 2011; 2012). Yet playing an essential role in living organisms as a micronutrient, when present at high concentrations Cu’s participation in Fenton reaction, which generates toxic ROS, is linked to cause several deleterious effects at different cellular levels (EPA, 2007; Maria and Bebianno, 2011). Cu concentrations in the aquatic system have been reported ranging from 0.03 to 0.23 $\mu g.L^{-1}$ in surface seawaters and from 0.20 to 30 $\mu g.L^{-1}$ in freshwater systems, additionally in water loads subjected to direct anthropogenic inputs concentrations can vary greatly reaching more than 100 $\mu g.L^{-1}$ (EPA, 2007).

In mussel species, exposure to environmental realistic concentrations of Cu were reported to affect its physiological status, namely inducing the alteration of several oxidative stress related antioxidant enzymes, LPO, immunomodulation, byssogenesis inhibition and metallothionein transcription expression and induction (Pipe *et al.*, 1999; Nicholson and Lam, 2005; Maria and Bebianno, 2011; Gomes *et al.*, 2011; 2012). Moreover, the results of mussels *M. galloprovincialis* exposure to realistic concentrations of Cu and polycyclic aromatic hydrocarbon benzo[a]pyrene (B[a]P) mixture which showed an overall higher alterations of antioxidant enzymes (SOD, CAT and GR) activities by 5 $\mu g.L^{-1}$ Cu single exposure (with the exception of Phase II GST) in both gills and digestive glands and LPO levels (in gills) than as a mixture component (Maria and Bebianno, 2011) were particularly relevant for its concentration selection and inclusion in one of the mixtures exposure assays (Chapter 6).

1.13 Objectives

Based on the several above mentioned introductory aspects, currently the knowledge regarding APIs occurrence and associated effects on non-target organisms is still very scarce, more so considering that the problematic is "emergent" by the scientific community. Nevertheless, in order to favor each API reliable associated ERA and subsequent associated regulatory measures, it is absolutely necessary to perform studies regarding validated methodologies of APIs quantification in all types of aquatic matrices and also provide data regarding the effects of each APIs, ideally through a multibiomarker assessment approach.

The objectives of this thesis pertained firstly, to contribute to the assessment of APIs concentrations in surface waters through the application of passive sampler (POCIS) and secondly, to the relevance of a multibiomarker approach application on the assessment of oxidative stress, neurotoxic and endocrine disruption effects using mussel *Mytilus galloprovincialis* as sentinel species. Based on the therapeutic classes of the APIs mentioned above one of the main objectives was to assess the effects of the selected APIs in mussel tissues comprising single exposure to environmental relevant concentrations and multiple stressors. Therefore specimens of *M. galloprovincialis* were collect from a reference site situated in the Ria Formosa Lagoon, Southeast of Portugal (37°06'58.62"N, 7°37'43.93"W)

This thesis is structured in seven Chapters organized as follows:

Chapter 1 Provides an introductory description concerning the problematic of APIs occurrence in the aquatic environment and potential effects on non-target organisms;

Which are the most important APIs occurring in Arade river? Is there any spatial and seasonal variability? These questions are addressed in **Chapter 2** where several APIs of therapeutic classes (analgesic, anticonvulsant, antidepressant, anxiolytic, antiasthmatic, anti-lipidemic, NSAID and stimulant) were identified and detected in Arade river applying the passive sampler POCIS with emphasis on summer impact changes.

Does non-steroidal anti-inflammatory (NSAID) ibuprofen affect oxidative stress responses and endocrine disruption in mussel *Mytilus galloprovincialis* tissues? **Chapter 3** address this problematic comprising a multibiomarker approach on antioxidant enzyme activities (SOD, CAT, GR and GST) alterations, damage (LPO) in

mussels gills and digestive gland and vitellogenesis (by ALP levels) in sex-differentiated gonads during the exposure to ibuprofen at an environmental relevant concentration (250 $ng.L^{-1}$).

Does selective serotonin reuptake inhibitor (SSRI) fluoxetine induce antioxidant system responses alteration, neurotoxic effect and/or endocrine disruption evidences in mussel *Mytilus galloprovincialis* tissues? This chapter (**Chapter 4**) focuses on the effects of an environmental relevant concentration of fluoxetine (75 $ng.L^{-1}$) in mussel tissues applying a multibiomarker approach on alterations in antioxidant enzyme activities (SOD, CAT and GST) in mussels' gills and digestive gland, AChE activity in gills and vitellogenesis (ALP) in sex-differentiated gonads.

Does NSAID diclofenac have the ability to alter antioxidant enzyme activities and produce neurotoxic and endocrine disruption effects in mussel *Mytilus galloprovincialis* tissues? **Chapter 5** focuses on the effects of anti-inflammatory diclofenac at an environmental relevant concentration (250 $ng.L^{-1}$) applying a multibiomarker approach on antioxidant enzyme activities (SOD, CAT, GR and GST) alterations, damage (LPO), neurotoxicity (AChE) and endocrine disruption (ALP) effects in mussels' gills, digestive gland, and sex-differentiated gonads.

Do distinct APIs mixtures induce distinct oxidative stress responses, neurotoxic and endocrine disruption effects in *Mytilus galloprovincialis*? What is the impact of a classic contaminant, such as Cu, in an emergent compound mixture? **Chapter 6** focuses on the effects of two mixtures of multiple stressors: one induced by a mixture of the APIs mentioned in the previous chapters and the other using the same mixture of emerging contaminants (APIs) with a classical contaminant (Cu). Effects on biomarkers of susceptibility (antioxidant enzymes SOD, CAT, GR and GST), damage (LPO), neurotoxicity (AChE) and endocrine disruption (ALP) were evaluated in mussels' gills, digestive gland and in sex-differentiated gonads and effects compared with those of the individual exposure.

Finally in **Chapter 7,** the overall conclusions were synthesized and future perspective studies proposed.

1.14 References

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Chapter 2

Occurrence of APIs in the aquatic system

2 Occurrence of APIs in the Aquatic System

Abstract

The development analytical techniques able to detect and quantify APIs in aquatic environment matrices is still recent and in constant evolution. Most techniques are very expensive, with complex technical challenges which depending on the circumstances only provide incomplete ("snap-shot") data concerning the concentration of APIs in a given time at a given site. There is almost no information concerning the actual APIs concentrations in Portuguese surface waters, this Chapter deals with the problematic around the detection of these emergent compounds and gives information about the usefulness of the application of passive samplers – like Polar Organic Compound Integrative Sampler (POCIS), which enable the assessment of time-weighted average (TWA) concentration of each bioavailable target analytes. Moreover, it constitutes the first study reporting the occurrence and quantification of APIs in Arade River (Portugal) encompassing a preliminary study made in 2009 to infer obtained data validity and a following study regarding the impact of Summer on the alteration of APIs concentrations in the same riverine water in 2010. The results reveal that out of the 21 APIs used as target analytes 14 were present in 2009 whereas in 2010 from the 19 target analytes 13 occurrent in Arade river although at different concentrations. In both cases, caffeine (CAF) was the active compound found at highest concentrations at both sites followed by the antiasthmatic theophylline (THEO). Although the summer impact is unclear, the results highlighted POCIS suitability for the quantification and profile of APIs concentrations in surface water following Water Frame Directive (WFD) recommendations.

2.1 Introduction

As mentioned in the previous chapter, pharmaceutical products have been used for centuries however, from an ecotoxicological viewpoint, APIs are considered emerging environmental contaminants since only recently the development of novel analytical and sampling techniques enabled their quantification in environmental samples (Söderström *et al.*, 2009; Munaron *et al.*, 2012). As APIs are designed to preserve their chemical structure to be therapeutically active, a serious concern about the environmental fate and

related impacts in water quality status and potential effects on non-target organisms has led to new EU and US legislative frameworks and directives (Roberts and Bersuder, 2006; Santos *et al.*, 2010). However APIs monitoring in natural waters is very complex due to APIs multiplicity in terms of e.g. physico-chemical properties, chemical structures and persistence in the environment (Castiglioni *et al.*, 2004; Togola and Budzinski, 2007a).

2.1.1 Active and Biological Sampling in Water Quality Monitoring

The often called conventional aqueous matrices active screening methods (like, grab and pump sampling) present two major limitations on an overall contaminant exposure assessment: 1) the ability to provide an accurate time integrative estimation only giving a snap-shot of contaminants concentrations at the exact sampling time often neglecting episodic environmental changes (such as accidental spills and seasonal variations) and 2) the ability to quantify target analytes at ultra-trace and/or trace levels in discrete water samples (Petty *et al.*, 2004; Alvarez *et al.*, 2005; Togola and Budzinski, 2007a; Arditsoglou and Voutsa, 2008; Söderström *et al.*, 2009). Even though these limitations can be respectively tackled by 1) the combination of multiple and repetitive time sampling and 2) large water sample volumes; these methodologies are extremely costly and time consuming involving large scale water samples clean-up, pre-treatment and pre-concentration processes (Arditsoglou and Voutsa, 2008; Söderström *et al.*, 2009; Togola and Budzinski, 2007a).

The estimation of water contaminants concentration applying biological samples through the analysis of contaminants in bivalve and fish tissues is based on species inherent contaminant bioconcentration processes and even though providing a much better understanding in terms of ecotoxicological risk assessment than discrete water sampling, this methodology is limited by several factors such as: 1) intra- and interspecific metabolism (e.g. detoxification mechanisms) and tested-organisms survival rate, 2) complex biological matrices chemical analysis and 3) relatively low uptake of polar organic compounds (POCs) compared to non-polar compounds in species tissues (Petty *et al.*, 2004; Söderström *et al.*, 2009).

2.1.2 Passive Sampling in Water Quality Monitoring

Passive samplers were conceived to tackle the difficulty of biological sampling data interpretation although mimicking biological uptake and concomitantly provide a time-weighted average (TWA) concentration of bioavailable target analytes reducing efficiently active sampling costs, maintenance and handling in water quality screening (Petty *et al.*, 2004; Alvarez *et al.*, 2005; Söderström *et al.*, 2009). For further knowledge the thoroughly reviews by Vrana *et al.* (2005) and Dévier *et al.* (2011) describing the broadly use, applications and current state of the art of passive sampling technology for waterborne organic and inorganic contaminants in environmental monitoring are recommended. In these studies two passive integrative samplers for the sequestering of ultra- to trace levels of organic contaminants mixtures in natural waters developed by US Geological Survey's Columbia Environmental Research Center (CERC) are referred: 1) the semipermeable membrane device (SPMD) (US Patent, 5,098,573, Huckins *et al.*, 1992; 5,395,426, Huckins *et al.*, 1995) for hydrophobic organic compounds and 2) the polar organic chemical integrative sampler (POCIS) (US Patent 6,478,961, Petty *et al.*, 2002) for hydrophilic organic compounds. Both passive samplers are based on the exchange flow kinetics of organic compounds from the surrounding water to the device receiving solid-phase by 1) partitioning for non-polar compounds trapped in SPMD and 2) by adsorption for polar compounds sequestered in POCIS, until the equilibrium is reached (Vrana *et al.*, 2005; Arditsoglou and Voutsa, 2008).

2.1.3 POCIS Concept and Modeling

POCIS devices are generally used for sampling polar organic molecules ($0 < \log K_{ow} < 4$) with no limitation of the type of natural waters being constituted by a solid receiving/sequestration phase (sorbent) enclosed between two hydrophilic microporous polyethersulfone (PES) membranes and attached by two stainless still rings (Petty *et al.*, 2002; 2004; Alvarez *et al.*, 2004; 2007; Vrana *et al.*, 2005) (Figure 2.1). The type of membranes used is optimal due to high analytes uptake rate and interfering macromolecules exclusion, minimal biofouling and durability compared to others like

Figure 2.1: Polar Organic Integrative Sampler (POCIS). Source: www.est-lab.com.

polysulfone having an average effective sampling surface of 41 cm^2 (Alvarez *et al.*, 2004; 2005). Furthermore, solely depending on the type of sorbent used POCIS can present two commercially available configurations: pesticide or more generic hydrophilic compounds-POCIS using a triphasic sorbent admixture of Isolute ENV+ polystyrene divinylbenzene (Argonaut Technologies, Redwood City, CA, USA) or pharmaceutical-POCIS applying Oasis hydrophilic-lipophilic balanced (HLB) (i.e. neutral) sorbent (Alvarez *et al.*, 2004; 2005). Since their development POCIS have been successfully applied in surface waters (lagoon, riverine and marine) and sewage effluents for the detection and quantification of numerous organic compounds like pharmaceuticals, polar pesticides, steroids, alkyphenols and hormones (Alvarez *et al.*, 2004; 2005; Jones-Lepp *et al.*, 2004; Petty *et al.*, 2004; MacLeod *et al.*, 2007; Mazzella *et al.*, 2007; 2010; Togola and Budzinski, 2007a; Arditsoglou and Voutsa, 2008; Zhang *et al.*, 2008; Harman *et al.*, 2008a, b; 2009; Martínez Bueno *et al.*, 2009; Li *et al.*, 2010; Pesce *et al.*, 2011; Munaron *et al.*, 2012).

The major drawback associated to the application of passive samplers particularly POCIS is the necessity of calculating accurate concentrations and availability of each target-compound sampling rate Rs ($L.day^{-1}$) pre-calibration data prior to its deployment in order to enable environmental water concentrations to be expressed as ng analyte per L (Togola and Budzinski, 2007a; Li *et al.*, 2010b; 2011). For this each polar analyte R_S has to be determined through laboratory experiments using flow-through or static renewal microcosm exposure systems describing the exchange kinetics between water and the passive samplers (Togola and Budzinski, 2007a; Söderström *et al.*, 2009). Nevertheless Rs depends on each compound physicochemical properties and environmental conditioning (e.g. water flow rate, temperature, pH, salinity, dissolved organic matter (DOM) and biofouling) (Roberts and Bersuder, 2006; Togola and Budzinski, 2007a; Söderström *et al.*, 2009; Li *et al.*, 2011). This limitation can be suppressed through the application of performance reference compounds (PRCs) like the ones added to receiving phase of SPMDs prior to deployment. PRCs release is afterwards measured and this difference enables *in situ* Rs appraisal (Huckins *et al.*, 2002). Although under investigation for application in POCIS this approach is still very costly (Togola and Budzinski, 2007a) and as argued by Munaron *et al.* (2012) POCIS exposed up to one month are unlikely to endure environmental parameter changes

(temperature, salinity and flow conditions) that would question the validity of Rs obtained by laboratory testing.

Contrarily with what was proposed by Alvarez *et al.* (2004) relating Rs value to the remaining analyte concentration in water after each exposure, Togola and Budzinski (2007a) refute that compound global loss in water is not exclusively due to analyte trapping in sorbent but also related to degradation kinetics phenomena.

Considering the above, the objective of this chapter pertains the detection and quantification of APIs concentrations in two different sites in Arade river, Portimão (Portugal) using passive sampler devices: POCIS. After the first screening of pharmaceutical compounds in the area in May/June 2009 representing one month POCIS deployment in both up and downriver sites, another survey on the same sites was carried out to assess the impact of tourism increase in the summer months (August to October) in 2010 on APIs concentration change due to the expected higher WWTPs discharges caused by the three-fold population density increase in the area during this period.

2.2 Materials and Methods

2.2.1 Sites Characterization

Located in Southern Portugal, Arade estuary comprises an area of 987 km^2 and is an important ecological and economic system in this region. Surrounded by saltmarsh riverbanks, Arade river cross several urban areas such as Ferragudo, Parchal, Mexilhoeira da Carregação and Portimão with an approximate total of 66,000 inhabitants (INE, 2010) and supports a marina and a large harbor. The major contamination sources derive mostly from municipal, industrial and agriculture (orcharding and rice culture) effluent discharges and runoff, but also from activities associated with tourism, marine traffic and aquaculture (DGPA, 2004; Fernandes *et al.*, 2007).

The two sites selected for POCIS deployment were (see Figures 2.2 and 2.3): Site 1 (also referred as downriver site) located inside Portimão commercial harbor (mostly dedicated to large cruises docking) on the west margin of Arade river and in the vicinity of two marinas and a shipyard, about 2 Km from the river mouth, (37º07’42.9”N,

8º31’50.8”W). Additionally, this site is under the influence to the North of Companheira WWTP discharges (WWTP1). This WWTP1serves Portimão city (55,614 inhabitants), Alvor (> 6000 inhabitants) and Mexilhoeira Grande (> 4000 inhabitants) (INE, 2011) to the west of Arade river and has been operating applying secondary treatment since 1982. Site 2 (also referred as upriver site) is about 2 Km upriver from site 1 and is located in the east margin of Arade river near a small recreational marina about 4 km from the river mouth (37º 08’54.1”N, 8º30’21.9”W). Additionally, this site is in very close vicinity of small Mexilhoeira da Carregação WWTP discharges (WWTP2, Figure 2.3).

Figure 2.2: POCIS deployment sites and WWTPs in Arade river. Source: ArcGIS 9 ArcMap version.

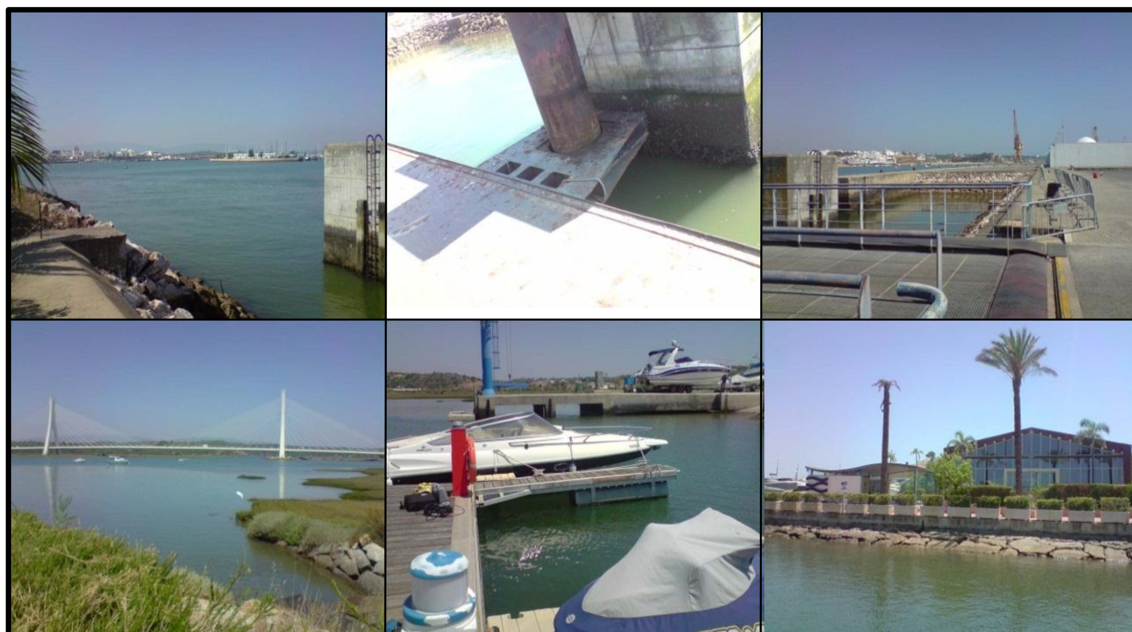

Figure 2.3: POCIS deployment in site 1 (up) and in site 2 (down).

2.2.2 POCIS Field Deployment

POCIS devices containing 200 mg Oasis HLB sorbent (exposure diameter of 54 mm and a sampling surface area of 45.8 cm^2) were from Exposmeter (Tavelsjö, Sweden) and provided by the Laboratoire de Physico- et Toxico-Chimie de l'Environnement (LPTC), Université de Bordeaux-1, Bordeaux (France), sent to the University of Algarve (Portugal) and immediately relocated at -20ºC.

First POCIS deployment took place for around a month from (25th May - 26th June 2009) under the project "PESSOA"; the second POCIS deployment took place from the 1st of August to the 1st of November 2010 under the project "PORTONOVO" in which by the end of each month POCIS were immediately collected and replaced by a new batch of POCIS. The devices were placed in triplicate inside a protective steel canister in the two sites as shown in Figure 2.4. The POCIS canister was tied with a rope and connected to the nearby structure at half-depth in the water column being stabilized with weight over the seabed. Temperature, salinity, pH and dissolved oxygen were measured with a multi-probe system (YSI 556MPS) (Table 2.1). By end of the exposure time (on average 30 days), each device was carefully rinsed with Milli-Q® ultrapure water and wrapped individually in aluminum foil and transported inside a cooler to the laboratory. Once in the laboratory POCIS were stored at -20ºC prior to analysis.

Figure 2.4: POCIS deployment in triplicate and protective cage.

Table 2.1: Abiotic parameters measurement per site per year

Year	Day/Month	Site	Temperature (°C)	Salinity	DO (%)	pH	depth
2009	25th May	1	18.1	33.7	122.0	7.9	2.5
	26th June		19.3	33.7	120.0	7.5	2.5
	25th May	2	20.3	30.7	149.0	7.6	1.5
	26th June		20.0	31.4	115.0	7.5	1.5
2010	6th August	1	23.8	34.5	105.4	7.9	2.5
	6th Setember		22.9	35.2	104.4	7.8	2.5
	6th October		16.3	37.1	104.0	7.9	2.5
	6th November		16.6	33.8	120.0	7.8	2.5
	6th August	2	24.6	34.1	88.5	7.9	1.5
	6th Setember		23.5	34.8	95.0	7.8	1.5
	6th October		16.4	33.9	98.8	7.8	1.5
	6th November		17.3	33.6	105.7	7.7	1.5

2.3 POCIS Content Analysis

2.3.1 Materials and Chemicals

Acetone, dichloromethane, acetonitrile and methanol (HPLC reagent grade, Scharlau) were purchased from ICS (Belin-Beliet, France). Glass SPE cartridges (6 mL) with Teflon frits (20 µm porosity) filled with Oasis HLB bulk sorbent (60 µm) were purchased from Supelco (Saint Quentin-Fallavier, France). Ultrapure deionized water was prepared using Milli-Q system (Millipore, Molsheim, France). All internal standards were purchased from Sigma Aldrich (St. Quentin Fallavier, France, purity > 98%).

2.3.2 POCIS Extraction

The extraction procedures for POCIS was adapted from Togola and Budzinski (2007a) and Tapie *et al.* (2011) with the intended target APIs (Table 2.2) time-integrated concentration assessment. Prior to analysis POCIS were removed from storage at -20ºC and allowed to reach room temperature. POCIS stainless still rings were then carefully disassembled (preventing the membrane perforation) and the two membranes detached and rinsed with ultrapure water to clean them in the best way possible of detritus avoiding any sorbent (solid-phase) loss (Figure 2.5).

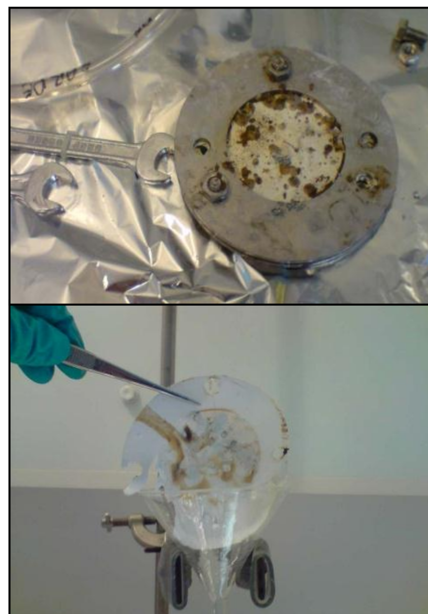

Figure 2.5: POCIS disassembling (up) and transferal of sorbent to SPE cartridge (down).

The sorbent was then directly transferred into previously weighted glass SPE cartridge with 5 ml of ultrapure water and dried by vacuum for 1 hour (until the frit inside the SPE cartridge was completely dried). A solution containing the internal standards (Table 2.3) was gravimetrically added to each sample and to the blanks and eluted using 10 ml methanol, 10ml 50:50 methanol/dichloromethane and 10 ml dichloromethane.

Table 2.2: Targeted-APIs

Therapeutic class	API/Common registred brand	CAS number
analgesic	aspirin **(ASP or ASA)***	50-78-2
	paracetamol Panadol **(PAR)**	103-90-2
anticonvulsant	carbamazepine Tegretol **(CAR)**	298-46-4
antidepressant	amitriptyline ADT **(AMI)**	50-48-6
	doxepin **(DOX)**	1668-19-5
	imipramine **(IMI)**	50-49-7
	fluoxetine Prozac **(FLX)**	54910-89-3
anxiolytic	alprazolam Xanax **(ALP)**	28981-97-7
	bromazepam Bromalex **(BRO)**	1812-30-2
	diazepam Valium **(DIA)**	439-14-15
	nordiazepam Sopax **(NOR)**	1088-11-5
NSAID	diclofenac Voltaren **(DCF)**	15307-86-5
	ibuprofen Brufen**(IBU)**	15687-27-1
	ketoprofen Profenid **(KET)**	22071-15-4
	naproxen Naprosyn **(NAP)**	22204-53-1
antiasthmatic	clenbuterol Mucopax **(CLE)**	37148-27-9
	salbutamol Ventilan **(SAL)**	18559-94-9
	terbutaline Bricanyl **(TER) ***	23031-25-6
	theophylline Eufilina **(THEO)**	58-55-9
anti-lipidemic	gemfibrozil Lopid **(GEM)**	25812-30-0
stimulant	caffeine **(CAF)**	58-08-2

APIs signaled by an * only screened in 2009

The cartridges were weighted again to assess sorbent mass. The flasks containing the resulting extract were then concentrated in a RapidVap vacuum evaporation system at 70% speed, 51ºC, vacuum 900 mbar for 25 minutes (this step of the procedure was not used on the 2009 samples) and finally evaporated to dryness applying a gentle nitrogen flux. Depending on the type electrospray ionization (ESI) required for each pharmaceutical, 150 µl of solvent was added and transferred into vials: acetonitrile for ESI- and a mixture of acetonitrile/water (v/v: 80/20) for ESI + and finally analyzed by liquid chromatography electrospray ionization tandem mass spectrometry (LC-MS-MS-ESI) using internal standard calibration (See Table 2.3). Also samples were blanks-corrected (n = 3) to detect potential contamination and recovery rates were assessed by the spiked samples. The recovery for the majority of the APIs was higher than 86%.

Table 2.3: Type of ESI ionization and respective internal standard used.

	API	Internal standard
ESI -	**ASP or ASA***	ibuprofen d3
	DCF	diclofenac d3
	IBU	ibuprofen d3
	GEM	gemfibrozil d6
	KET	ketoprofen d3
	NAP	naproxene d3
ESI +	**AMI**	amitriptyline d6
	ALP	diazepam d5
	BRO	diazepam d5
	CAF	caffeine c13
	CAR	diazepam d5
	CLE	diazepam d5
	DIA	diazepam d5
	DOX	amitriptyline d6
	FLX	amitriptyline d6
	IMI	amitriptyline d6
	NOR	nordiazepam d5
	PAR	paracetamol d4
	SAL	salbutamol d3
	TER*	diazepam d5
	THEO	diazepam d5

API signaled by * only analyzed in 2009

2.3.3 Calculation of APIs Concentration

According to Togola and Budzinski (2007a) the R_s should be calculating by the following equation (1):

$$Rs = \frac{\frac{Qs}{Q0}}{t}V \qquad (1)$$

Rs - sampling rate

Qo - initial analytes quantity in water

Qs - analyte quantity in sorbent at the end of exposure

V - volume of water (L)

t - exposure time in days

Applied Rs (L. day^{-1}) for concentration assessment followed the ones obtained by Togola (2006), Togola and Budzinski (2007a), Budzinski *et al.* (2009) and MacLeod *et al.* (2009).

The concentration of each analyte in natural water samples was calculated applying the following the equation (2):

$$Cw = \frac{Cs.Ms}{Rs.t} \qquad (2)$$

Cw - concentration in water

Cs - concentration in sorbent

Ms - mass of sorbent

Rs – sampling rate

t – exposure time in days

2.3.4 Statistical Analysis

Two-way ANOVA was performed using SIGMAPLOT® to test differences between each API concentration per site per month. When needed ad-hoc Holm-Sidak was used on single API differences discrimination over time within and between sites.

Principal component analysis (PCA) was performed using XLSTAT® 2012 to assess each targeted-analyte and abiotic parameters responsibility on the variability at each set of time (by month) in both sites within 2010. Statistical significance was defined at $p < 0.05$.

2.4 Results

Neither DOX, CLE, IMI, KET or SAL were not detected between years, while AMI and BRO were only not detected in 2009[2] and 2010 respectively.

2.4.1 First Screen

In the POCIS deployed in 2009, from the 21 targeted analytes, 14 were detected between sub- and ng.L^{-1} range (Figures 2.6 and 2.7).

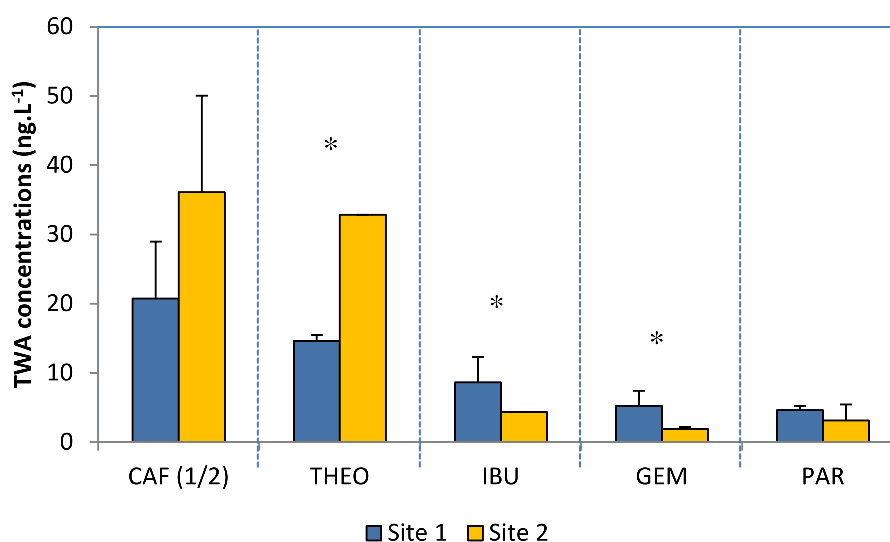

Figure 2.6: TWA concentrations (ng.L^{-1}) in two sites of the Arade river in 2009. CAF concentration scale was divided by a factor of 2. * highlights differences between sites ($p < 0.05$).

The highest APIs concentrations (Figure 2.6) decreased in the following pattern: for site 1 (downriver) - CAF > THEO > IBU > GEM > PAR and for site 2 (upriver) - CAF > THEO > IBU > PAR > GEM. For the lowest concentrations (Figure 2.7) decrease pattern were: for site 1 – NAP > CAR > NOR > DCF > BRO > ALP > DIA[3]; for site 2 – NAP > CAR > DCF > FLX > NOR > ALP > DIA and only BRO was below detection limit. For the top detected analytes CAF and THEO concentrations were 1.7- and 2.2-fold higher at site 2 although only significantly for THEO, while inversely

[2] ASP and TER were not considered in the results since the recovery rate was below 35% and therefore not included in 2010 assessment.
[3] FLX was below detection limit

concentrations of IBU, GEM and PAR were 2.0-, 2.7- and 1.5-fold higher in site 1 only not significantly for PAR. Concerning the APIs detected at lower concentrations, NAP, CAR, DCF were similar at both sites while NOR, BRO and DIA were higher at site 1 and ALP and FLX t site 2.

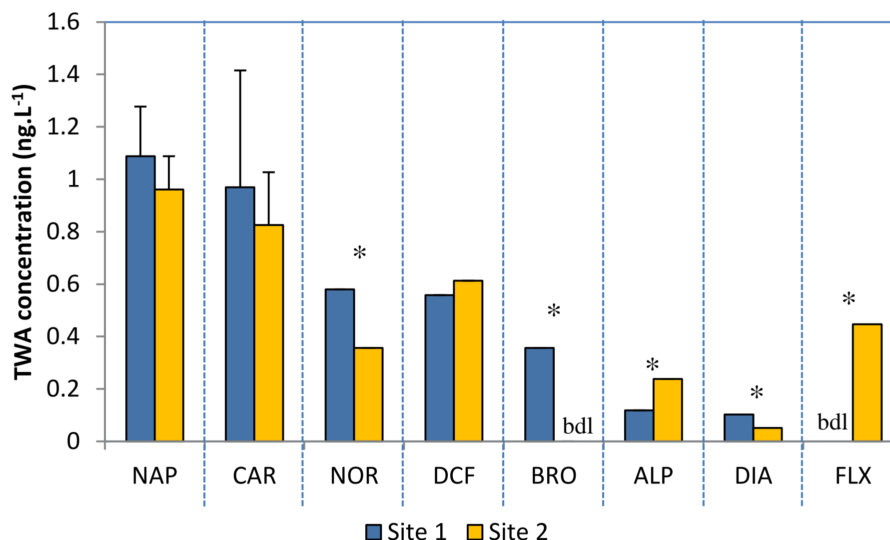

Figure 2.7: TWA concentrations (ng.L^{-1}) in two sites of the Arade river in 2009. bdl = below detection limit. * highlights differences between sites ($p < 0.05$).

Comparing the percentage of therapeutic classes between both sites (Figures 2.8A and B), the overall pattern is similar. Nevertheless, there were no antidepressants at site 1 (concentrations below detection limit) (Figure 2.8A) and the percentage of anti-lipidemics (a.k.a. lipid-regulators) (gemfibrozil), analgesics (paracetamol), NSAIDs, anticonvulsants and anxiolytics was higher than at site 2 (Figure 2.8B). Conversely, the percentage of stimulants (CAF) and antiasthmatics (theophylline) was higher at site 2. Regarding therapeutic classes without stimulants (CAF) (Figures 2.8C and D) it is evident the prevalence of antiasthmatics at site 2 (32% higher than at site 1), which in turn has approximately twice the percentage of NSAIDs. This is followed by a switch of anti-lipidemics and analgesics between sites, 10 and 6% higher respectively at site 1. Moreover the distribution of therapeutic classes' was more homogeneous at site 1 which apart from antiasthmatics was always consistently higher at site 2.

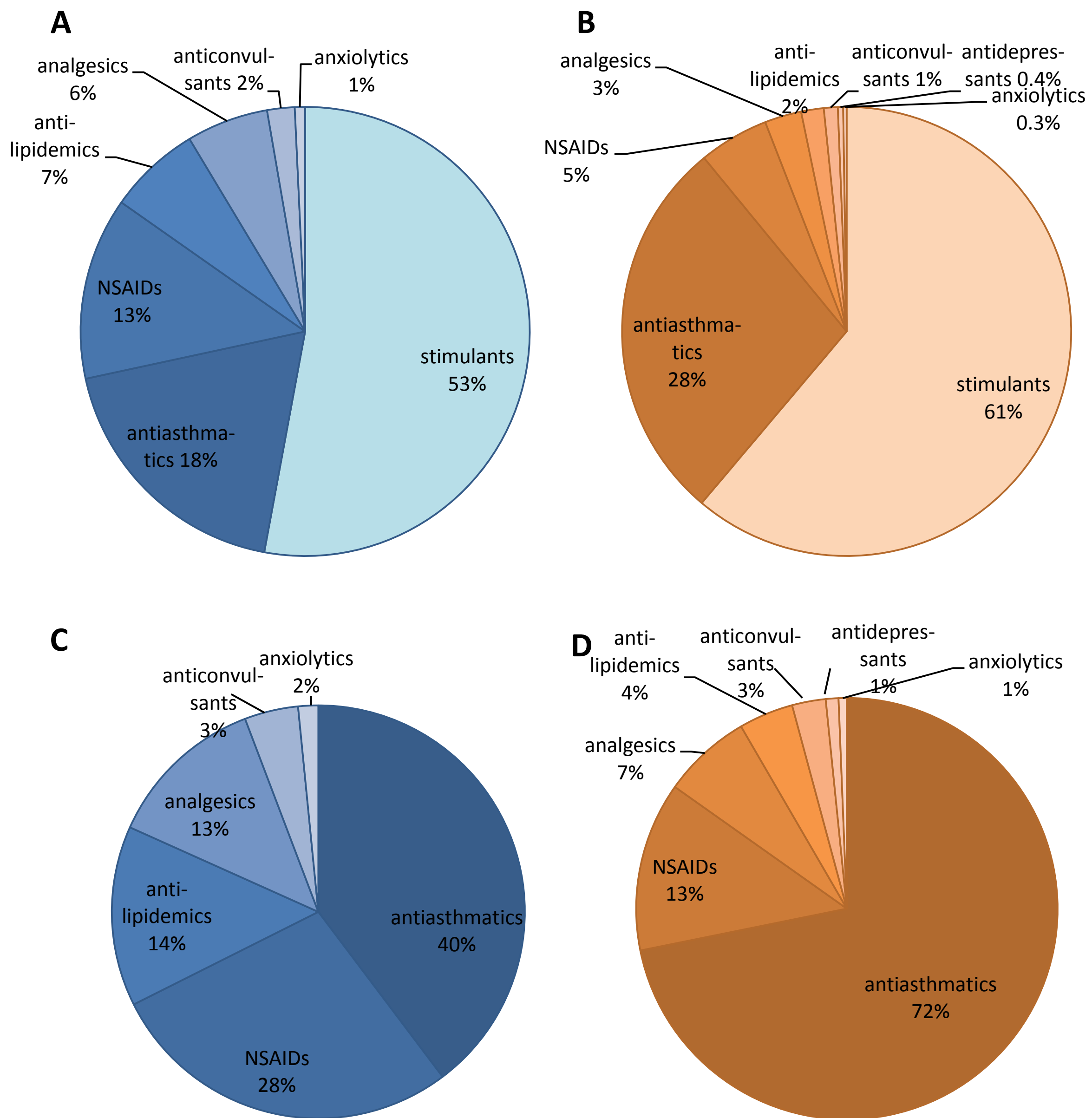

Figure 2.8: Percentage of APIs in Arade river in 2009 according to therapeutic classes + stimulant (CAF) (A - site 1 downriver, B - site 2 upriver) and therapeutic classes without CAF (C - site 1 downriver, D – site 2 upriver).

2.4.2 Evolution of APIs Concentrations

In the POCIS deployed in 2010, from the 19 targeted analytes, 13 were detected at sub- and ng.L^{-1} range whose variation was site and time-dependent. The highest concentration detected was that of CAF (Figure 2.9) with an overall average 804 ± 209 ng.L^{-1}, showing no significant differences between sites or months, although at site 2 there was a tendency for a concentration increase with time ($p > 0.05$). The antiasthmatic THEO followed and in site 1 THEO concentrations significantly decreased after the first month increasing again in October while on site 2 there was an increase until September showing similar concentrations over the following months (maximum concentration of 186 ± 44 ng.L^{-1}). Moreover, THEO concentrations although similar at both sites in the beginning, became significantly higher at site 2 in September and October ($p < 0.05$).

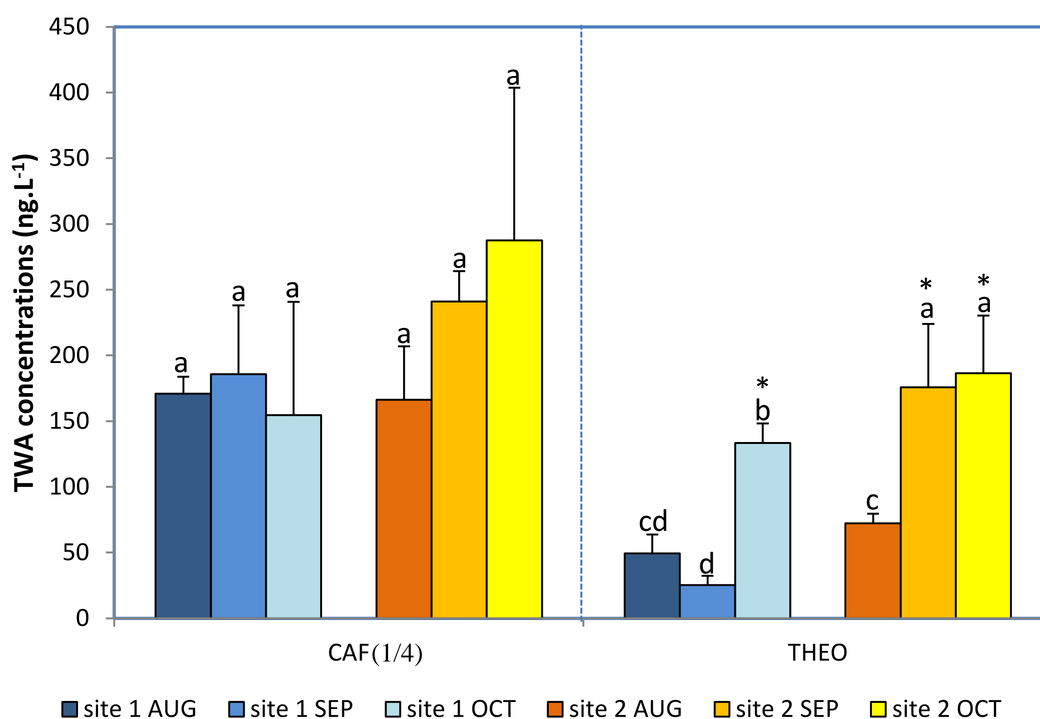

Figure 2.9: TWA concentrations (ng.L^{-1}) (average ± SD) of CAT and THEO in Arade river sites between August to October 2010 (n = 3). CAF concentration was divided by a factor of 4. Different letters indicate significant differences within each API, * highlights differences between sites ($p <$ 0.05).

The following APIs detected were analgesic (PAR), anticonvulsant (CAR), NSAIDs (IBU, DCF and NAP) and anti-lipidemic (GEM) ranging from 3 to 88 $ng.L^{-1}$ (Figure 2.10).

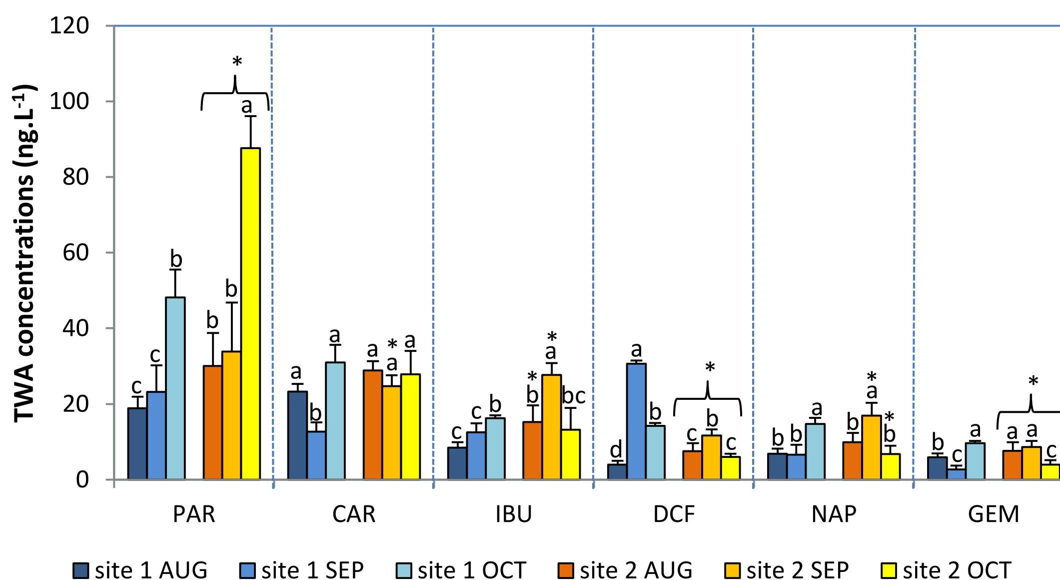

Figure 2.10: TWA concentrations ($ng.L^{-1}$) (average ± SD) of PAR, CAR, IBU, DCF, NAP and GEM in Arade river sites between August to October 2010 (n = 3). Different letters indicate significant differences within each API, * highlights differences between sites ($p < 0.05$).

PAR concentration varied similarly in both sites showing an enhancement tendency over time, although similar between August and September. Both sites showed a clear increase during October but 1.8-fold higher at site 2 reaching a maximum of 88 ± 8 $ng.L^{-1}$ ($p < 0.05$). CAR concentration was quite similar in both sites. Even though CAR at site 1 showed a decrease in September (significantly lower than October) no significant differences were found between sites (maximum of 31 ± 5 $ng.L^{-1}$ in site 1). IBU concentration increased progressively over time at site 1 although only significantly by the end of October, while at site 2 the highest concentration was in September and decreased in October to levels similar to the beginning ($p < 0.05$). Comparing both sites, IBU was significantly higher in August and September in site 2. DCF concentrations followed the same pattern at both sites, showing a steep increase from August to September followed by a significant decrease in October with significantly higher DCF concentration in September at site 1. The comparison between sites showed a transient pattern while higher concentrations at site 2 in August, but over the following months, site 1 showed significantly higher DCF concentrations. NAP concentrations only increased significantly in October in site 1 being significantly

higher than in site 2. At site 2, NAP concentrations in the beginning were similar to site 1 and increased in September reaching levels similar to August in October ($p < 0.05$). Finally, GEM concentrations varied significantly over time in site 1, decreasing between August and September reaching a maximum in October that was similar to levels detected in August and September at site 2. In site 2 the only significant difference was a GEM decrease over October.

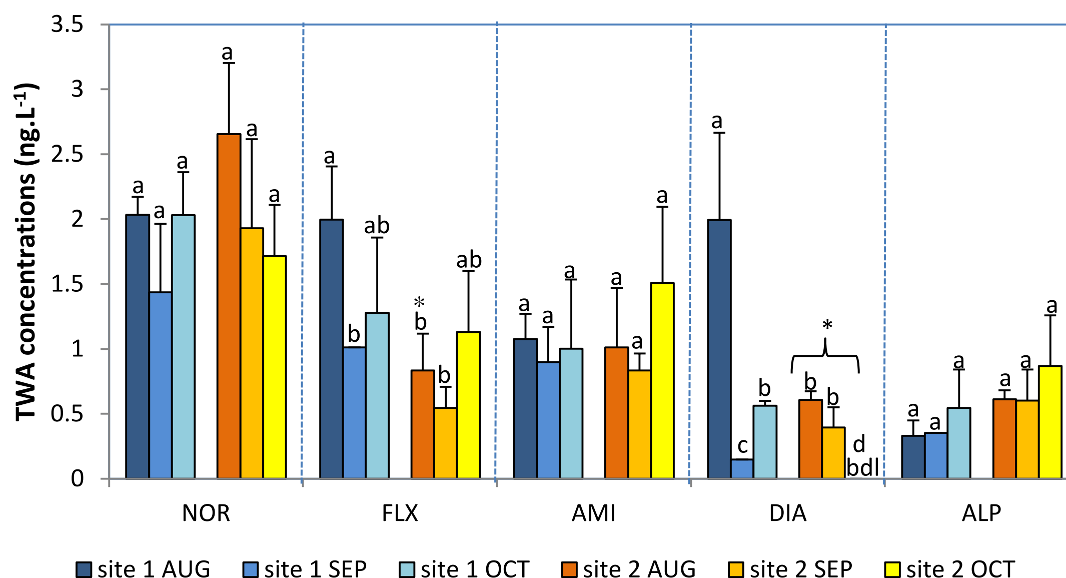

Figure 2.11: TWA concentrations (ng.L^{-1}) (average ± SD) of NOR, FLX, AMI, DIA, ALP in Arade river sites between August to October 2010 (n = 3). Different letters indicate significant differences within each API, bdl = below detection limit. * highlights differences between sites (*p*

The lowest APIs concentrations detected included anxiolytics and antidepressants (NOR, FLX, AMI, DIA and ALP) with concentrations below 3 ng.L^{-1} (Figure 2.11). NOR concentrations did not vary significantly between sites or time, although levels at site 2 were slightly higher than at site 1 and showed a decrease tendency with time ($p > 0.05$). The pattern variation of FLX concentrations was similar between sites. However, levels were only significantly higher in August at site 1. AMI concentrations were also not site or time dependent. Moreover, DIA concentration was significantly different in site 1; the highest concentration was in August followed by an abrupt decrease in September increasing again by October. In site 2, DIA concentration showed a progressive decrease reaching a concentration below detection limit in October. Furthermore, DIA was significantly higher at site 1 than at site 2 in August and October. Finally, ALP concentrations showed like NOR and AMI, to be not site nor time dependent.

In terms of therapeutic classes with stimulants (Figures 2.12A and B), CAF has in both sites a similar percentage 77% (site 2) to 80% (site 1) of overall targeted analytes detected. Compared to the first screen in 2009 (Figures 2.8A and B) CAF in 2010 is more prevalent in site 1 rather than site 2. Antiasthmatics are again the API more prevalent in site 2 (12%) than in site 1 (8%) as observed in 2009, although with lower percentage. Inversely to the homogeneity found in 2009 in terms of NSAIDs distribution (also in higher percentages than 2010), site 2 has a higher percentage of analgesics than NSAIDs which in this case is opposite of what was found in 2009. Furthermore, in 2010 anticonvulsants had higher prevalence in both sites than anti-lipidemics, contrarily of what was observed in the first screen. Finally the overall percentage of anxiolytics and antidepressants, in both sites in 2010 was very similar, whether in 2009 no antidepressants were found in site 1. In terms of APIs percentages reported without CAF (Figures 2.12C and D), the percentage of antiasthmatics in site 1 was similar with the one in 2009, being again lower than at site 2. The same can be said for NSAIDs in site 1 between years. However in site 2 analgesics were 2-fold higher in 2010 than 2009, being lower than NSAIDs. For the following therapeutic classes, while in site 1 in 2010 anti-lipidemics are 10% lower than in 2009 and site 2 exhibits a similar percentage between years, anticonvulsants are 10% and 7% higher in site 1 and 2 over 2010 than in 2009.

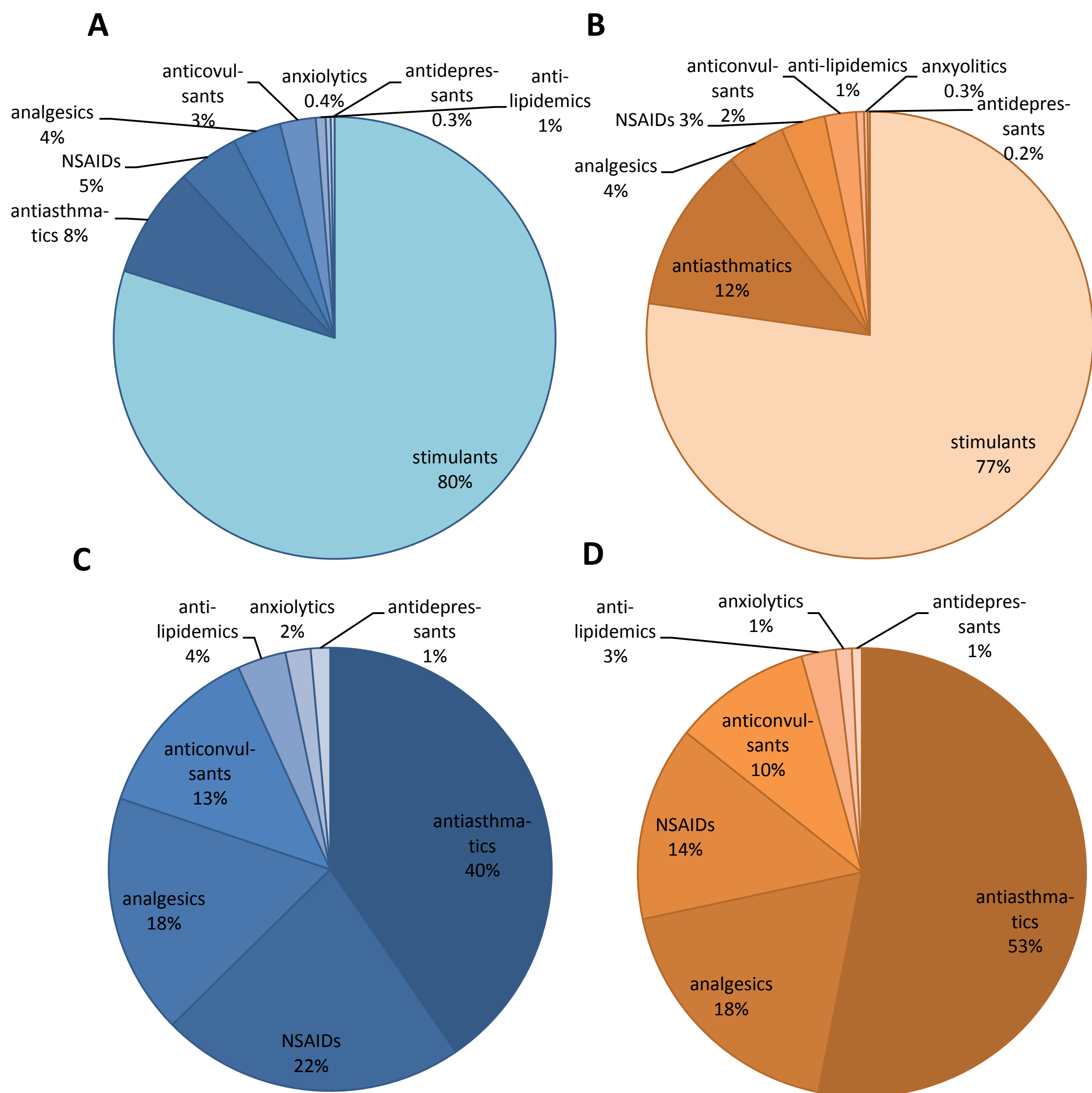

Figure 2.12: Percentage of APIs in Arade river in 2010 according to therapeutic classes + stimulant (CAF) (A– site 1 downstream, B – site 2 upstream) and therapeutic classes without stimulant (CAF) (C – site 1 downstream, D – site 2 upstream).

2.4.3 Principal Component Analysis (PCA)

PCA was applied to the average of all target-analytes concentrations present at both sites in 2010 (Figure 2.13). PCA expresses 61% of Total Variance (Tvar) highlighting a separation within site 1 in which August distribution is further related to temperature and DIA, FLX while September is associated to salinity, pH and DCF which ultimately contrast with October dispersal further associated to NOR, GEM, CAR and AMI.

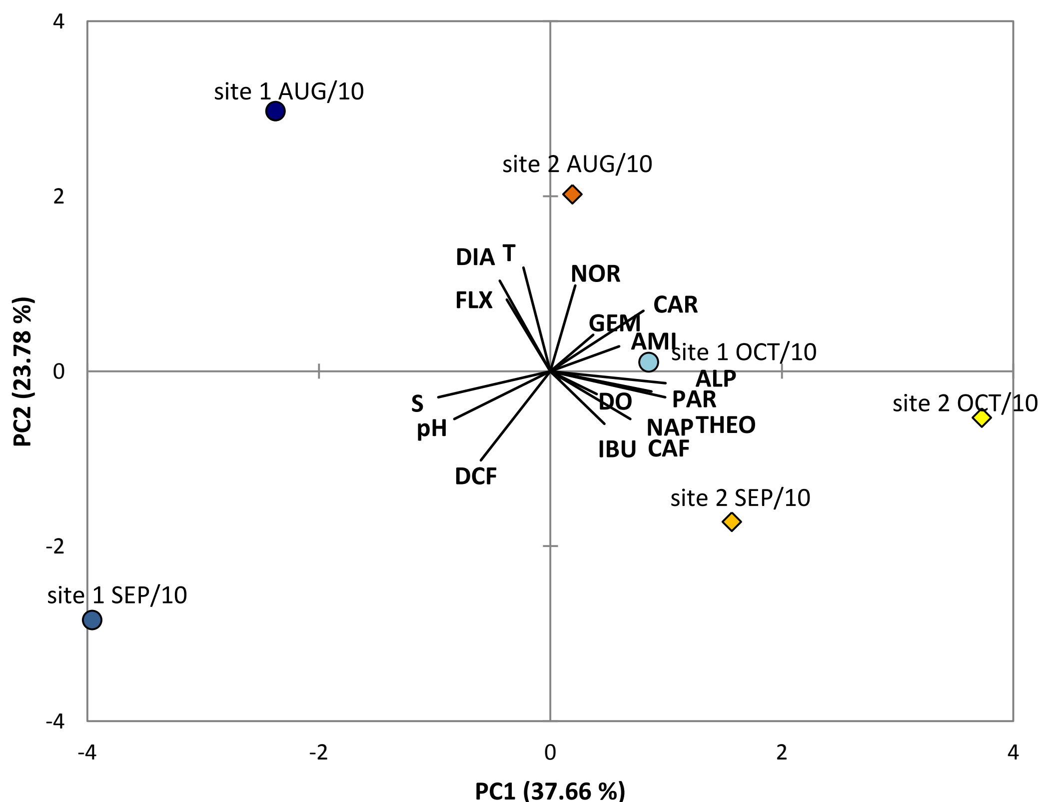

Figure 2.13: PCA representing 61% of total variance of targeted-analytes concentration integration between sites and time from Arade river 2010 (● – site 1 and ♦ – site 2, color scheme is in agreement with the one previous applied in Figures above).

At site 2, overall month dispersal is less noticeable than at site 1. Nevertheless August is further associated with NOR, GEM, CAR and AMI while the higher proximity among September and October is affected by the dispersal of NSAIDs (NAP, IBU) and CAF distribution with ALP, PAR and THEO respectively. Overall, site 2 seems to be more affected by APIs distribution, than unlike site 1 further dependent of abiotic parameters distribution and antidepressants and anxiolytics presence.

2.5 Discussion

The assessment of average concentrations of contaminants in water bodies is one of the pivotal goals covered by the WFD for this reason the application of passive samplers that enable TWA concentrations determination provide a more holistic approach of water quality status compared to spot sampling (Dévier *et al.*, 2011; Morin *et al.*, 2012). The present results identify for the first time the presence of APIs screening by POCIS devices in Portugal and in the Arade river. Being one of the very few studies focusing on this subject in Portuguese water bodies apart from the ones made in Douro river analyzed by liquid chromatography–ion trap tandem mass spectrometry after off-line solid-phase extraction (SPE) (Madureira *et al.* 2009; 2010) analogous to POCIS solid-phase medium which targeted 7 APIs analytes (antibiotics - trimethoprim and sulfamethoxazole, beta-blocker - propranolol, lipid regulating agent - fenofibric acid and FLX, CAR, DIA common with the present study).

In overall, 2009 TWA APIs concentrations were lower than 2010 except for BRO (which was not detected in the following year), IBU and GEM that presented both concentrations similar between both years in site 1. Nevertheless this scenario may have been influenced by the different extraction methodology applied between years rather than environmental conditioning (abiotic parameters) variation, thus not justifying Rs selection alteration. In 2010 results revealed good reproducibility amongst POCIS triplicate, with an analyte accumulation variability averaging approximately 30% in each site. APIs recovery rates were also in the range from 84 to 110 % .

2.5.1 Summer Impact in APIs Concentrations in Arade River

Regarding the impact of summer months in APIs concentrations assessment in the Arade river the results were quite inconclusive and transient between months in both sites. Nevertheless, at site 1 August presented higher concentrations of antidepressants (FLX and AMI) and anxiolytic (DIA), September higher concentrations of stimulant (CAF) and NSAID (DCF) and finally October presented the highest concentrations of several therapeutic classes such as antiasthmatic (THEO), analgesic (PAR), anticonvulsant (CAR), NSAIDs (IBU and NAP), anti-lipidemic (GEM) and anxiolytic (ALP). In general at this site, September was the month exhibiting lower concentrations of APIs whereas October exhibited the highest. Moreover, PAR, IBU and ALP were the

only APIs which nominal concentrations progressively increased over time, whereas the inverse was never observed.

At site 2 the dissimilarity of APIs concentration distribution between months was less pronounced than at site 1. Nevertheless anticonvulsant CAR and anxiolytics NOR and DIA concentrations were slightly highest in August ($p > 0.05$). September showed significantly higher concentrations of NSAIDs IBU, DCF (alike site 1) and NAP and anti-lipidemic GEM. Finally alike site 1, October was globally the month showing higher APIs concentrations such as stimulant (CAF), antiasthmatic (THEO), analgesic (PAR), antidepressants (FLX and AMI) and anxiolytic (ALP) of which THEO, PAR and ALP concentrations were concomitantly higher at both sites at this month. Moreover CAF, THEO and PAR which were the APIs with overall highest concentrations in Arade river in both sites, showed a progressive enhancement with time at site 2, while anxiolytics NOR and DIA showed the opposite, decreasing over time. Finally, the three months average revealed that site 2 had consistently the highest APIs concentrations (with the exception of DCF, FLX and DIA) which can be explained by the closer proximity of WWTP2 discharge, higher site confinement and thus longer water renewal time than site 1 which is at greater distance of WWTP1. Moreover, the overall presence of APIs concentrations throughout summer (dry-season) particularly by October also attests to uncontrolled WWTPs discharges since as stated by Vystavna *et al.* (2012) water run-off volume at this period is usually low.

2.5.2 Comparison with other TWA Concentrations in Surface Waters

Very few studies have yet applied POCIS devices on the TWA concentration determination of APIs target-analytes. Table 2.4 presents the up to date TWA maximum concentrations reported of the selected APIs with the present study. In comparison with the maximum TWA concentrations found between different up and downstream of WWTPs sites in Lopan river (Ukraine) and Jalle river (France) targeting exactly the same analytes (Vystavna *et al.*, 2012), Arade river presented similar concentrations of NOR, FLX and AMI than Jalle river all APIs occurring at a maximum concentration below 2 $ng.L^{-1}$, while Lopan river did not present any concentration of NOR and 5-fold higher concentrations of FLX and AMI. Alike Arade river ASP, BRO, CLE, DOX, IMI, SAL and TER were also not detected in both Ukrainian and French rivers. Furthermore and only concerning the maximum concentration reported, Arade river presented 1.7

and 8-fold higher concentrations of PAR and CAF than Lopan river and 1.9 and 5-fold higher concentrations of IBU and GEM than Jalle river, while CAR concentration was 9-fold lower than Lopan river and DCF and NAP were more than 2-fold lower than Jalle river. Unlike Arade river THEO, DIA and ALP concentrations were not present in neither Lopan nor Jalle rivers, while KET concentration which was not detected in Arade river was present in both rivers (60 and 130 $ng.L^{-1}$ respectively).

In a coastal lagoon and semi-enclosed bay in French Mediterranean coast (Munaron *et al.*, 2012) lower CAF concentrations in all sites (up to 32 $ng.L^{-1}$), also CAR (up to 12 $ng.L^{-1}$) and GEM (up to 5 $ng.L^{-1}$) which was only present in the semi-enclosed lagoon site, than the ones found in both sites in Arade river. Like reported by Vystavna *et al.* (2012), KET was also found up to 3.5 $ng.L^{-1}$ unlike for Arade river while all the remaining targeted APIs such as DIA and DCF were only found at ultra-trace levels (< 1 $ng.L^{-1}$). The presence of higher concentrations of APIs in more confined waters at lagoon and bay sites when compared to coastal water sites was justified due to longer water renewal time associated with higher urbanized watersheds (Munaron *et al.*, 2012), as observed for Arade river site 2 compared to site 1.

Lastly, Arade river presented approximately 5 and 14-fold lower concentrations of DCF and CAR than Ouse river in the United Kingdom (Zhang *et al.*, 2008) and 7-fold lower FLX than Crabtree Creek in USA (Bringolf *et al.*, 2010).

Table 2.4: TWA concentrations of commonly detected APIs in surface waters using POCIS

Therapeutic class	API	Analytical Method	Maximum concentration (ng.L^{-1})	Location	Reference
stimulant	**CAF**	POCIS Oasis HLB/ LS-MS-MS-ESI	32	Mediterranean French coast (France)	Munaron *et al.*, 2012
			80	Jalle river (France)	Vystavna *et al.*, 2012
			150	Lopan river (Ukraine)	
			1150	**Arade River (Portugal)**	**present study**
anticonvulsant	**CAR**	POCIS Oasis HLB/ LS-MS-MS-ESI	12	semi-enclosed lagoon (France)	Munaron *et al.*, 2012
			25	Jalle river (France)	Vystavna *et al.*, 2012
			31	**Arade River (Portugal)**	**present study**
			275	Lopan river (Ukraine)	Vystavna *et al.*, 2012
		POCIS Oasis HLB/ LS-MS-MS	427	Ouse river (United Kingdom)	Zhang *et al.*, 2008
analgesic	**PAR**	POCIS Oasis HLB/ LS-MS-MS-ESI	45	Jalle river (France)	Vystavna *et al.*, 2012
			50	Lopan river (Ukraine)	
			88	**Arade River (Portugal)**	**present study**
NSAIDs	**IBU**	POCIS Oasis HLB/ LS-MS-MS-ESI	15	Jalle river (France)	Vystavna *et al.*, 2012
			28	**Arade River (Portugal)**	**present study**
	DCF	POCIS Oasis HLB/ LS-MS-MS-ESI	<10	Lopan river (Ukraine)	Vystavna *et al.*, 2012
			31	**Arade River (Portugal)**	**present study**
			70	Jalle river (France)	Vystavna *et al.*, 2012
		POCIS Oasis HLB/ LS-MS-MS	165	Ouse river (United Kingdom)	Zhang *et al.*, 2008
	NAP	POCIS Oasis HLB/ LS-MS-MS-ESI	< 10	Lopan river (Ukraine)	Vystavna *et al.*, 2012
			17	**Arade River (Portugal)**	**present study**
			40	Jalle river (France)	Vystavna *et al.*, 2012
anti-lipidemic	**GEM**	POCIS Oasis HLB/ LS-MS-MS-ESI	< 2	Jalle river (France)	Vystavna *et al.*, 2012
			5	semi-enclosed lagoon (France)	Munaron *et al.*, 2012

Table 2.4: (Continuation).

anti-lipidemic	**GEM**	POCIS Oasis HLB/ LS-MS-MS-ESI	**10**	**Arade River (Portugal)**	**present study**
antidepressant	**FLX**	POCIS Oasis HLB/ LS-MS-MS-ESI	**< 2**	**Arade River (Portugal)**	**present study**
			< 2	Jalle river (France)	Vystavna *et al.*, 2012
			< 10	Lopan river (Ukraine)	
			14	Crabtree Creek (USA)	Bringolf *et al.*, 2010
	AMI	POCIS Oasis HLB/ LS-MS-MS-ESI	**<2**	**Arade River (Portugal)**	**present study**
			<2	Jalle river (France)	Vystavna *et al.*, 2012
			<10	Lopan river (Ukraine)	
anxiolytic	**NOR**	POCIS Oasis HLB/ LS-MS-MS-ESI	< 2	Jalle river (France)	Vystavna *et al.*, 2012
			3	**Arade River (Portugal)**	**present study**

2.5.3 Arade River API Concentrations in Comparison with other Studies Applying Spot Sampling in Surface Waters

An overview of the commonly selected and detected APIs concentrations in surface waters obtained using spot sampling is in Table 2.5. Douro river (Madureira *et al.*, 2009; 2010) (downstream of WWTP discharge) presented concentrations of CAR (178 $ng.L^{-1}$) and DIA (3.7 $ng.L^{-1}$) in June 2008 higher than the ones found in Arade river in 2009 and 2010: 1 and 31 $ng.L^{-1}$ of CAR (site 1 in June 2009 and October 2010 respectively) and 0.1 and 2 $ng.L^{-1}$ of DIA (site 1 in June 2009 and August 2010 respectively), whereas FLX was only detected in the Arade river 0.5 and 2 $ng.L^{-1}$ (site 2 June 2009 and site 1 in August 2010 respectively while in Douro river was not quantified due to lack of accuracy upon validation process. In this particular case, even though the urban areas adjacent to Arade river comprise only an approximate average of 65,000 inhabitants having 8-fold lower population density than Douro River estuary in the vicinity of Oporto (237,584 inhabitants) and Vila Nova de Gaia (302,296 inhabitants) cities (INE, 2011) it is clear that this riverine water is as vulnerable to APIs occurrence, although is expected that the different sampling techniques used may induce proportionally higher values at Arade river due to TWA concentrations given by POCIS.

Furthermore, in an overall comparison of the results with spot sampling (Table 2.5), Arade river presented high concentrations of CAF (max. of 1150 $ng.L^{-1}$) and PAR (max. of 88 $ng.L^{-1}$) being in agreement with the ones found in Halifax watershed (Comeu *et al.*, 2008) and Ebro river basin (Gros *et al.*, 2010) respectively which in turn comprise highly populated areas of approximately 390,000 and 3 million inhabitants (Canada Statistics, 2012; Deltanet Project, 2012). Concomitantly presented similar concentrations of CAR (max. of 31 $ng.L^{-1}$ in Arade river) with Tamis river in Serbia (Vasiljevi *et al.*, 2009); IBU (max. of 28 $ng.L^{-1}$ in Arade river) with Zhujiang river (China) (Zhao *et al.*, 2010) and Elbe river (Germany) (Weiger *et al.*, 2004); DCF (max. of 31 $ng.L^{-1}$ in Arade river) with Lake Alster (Germany) (Weiger *et al.*, 2004) and Zhujiang river (China) (Zhao *et al.*, 2010); NAP (max. of 17 $ng.L^{-1}$ in Arade river) with Lake Greifensee (Switzerland) (Öllers *et al.*, 2001) and Llobregat river (Spain) (Farré *et al.*, 2001); GEM (max. of 9 $ng.L^{-1}$ in Arade river) with Zhujiang river (China) (Zhao *et al.*, 2010) and Halifax watershed (Canada) (Comeau *et al.*, 2008).

It is obvious that APIs presence in water is dependent of social-economic-demographic factors and prescription practices of each region yet and foremost these emergent compounds entrance in the environment is due to inefficient WWTP treatments. Our results highlight the necessity of WWTPs treatments improvement in order to avoid further Arade river contamination by these compounds. The high presence of CAF, which is known to have a non-conservative behavior and be easily degradable during WWTP treatments and in aquatic systems (Togola and Budzinski, 2007a, Vystavna *et al.* 2012) and PAR concentrations compared to the above mentioned studies are particularly concerning since as remarked by Madureira *et al.* (2010) even though the presently detected APIs concentrations are not individually pertained to cause acute effects, it should not be excluded the potential chronic risks/effects of exposure to a continuous discharge of APIs mixtures in the ecosystem especially on commercial and economically relevant non-target species like bivalves (see following Chapters).

Table 2.5: Maximum concentrations of the commonly detected APIs in surface waters collected by spot sampling

Therapeutic class	API	Country	Location	Analytical Method	Maximum concentration ($ng.L^{-1}$)	Reference
stimulant	CAF	France	Lergue river	Oasis MCX, C18, HLB/ GC-MS	107	Togola and Budzinski, 2008
			Seine estuary	Oasis HLB/ GC-MS	138	Togola and Budzinski, 2007b
			Cortiou rocky inlet	Oasis MCX, C18, HLB/ GC-MS	8000	Togola and Budzinski, 2008
		Germany	Elbe river	Oasis HLB/ GC-MS	148	Weigel *et al.*, 2004a
			Lake Alster	Oasis HLB/ GC-MS	176	
		Romania	Somes river	Oasis HLB/ GC-MS	9700	Moldovan, 2006
		Spain	Henares-Jarama-Tajo river	Oasis HLB/ HPLC-MS-ESI	416	Fernández *et al.*, 2010
		Canada	Detroit river	Oasis HLB/ HPLC-MS	40	Hua *et al.*, 2006
			Halifax watershed	Oasis HLB/ GC-MS	1100	Comeau *et al.*, 2008
		USA	Salt Creek	Oasis HLB/ GC-MS	18	Bartelt-Hunt *et al.*, 2009
			Tennessee river	Oasis HLB/ UPLC	32	Conley *et al.*, 2008
			Wood River	Oasis HLB/ GC-MS	321	Bartelt-Hunt *et al.*, 2009
			Big Blue river	Oasis HLB/ GC-MS	980	
anticonvulsant	CAR	China	Liuxi river	Oasis HLB/ GC-MS	18	Zhao *et al.*, 2010
			Zhujiang river	Oasis HLB/ GC-MS	26	
			Shijing river	Oasis HLB/ GC-MS	43	
		France	Lergue river	Oasis MCX, C18, HLB/ GC-MS	56	Togola and Budzinski, 2008
			Seine estuary	Oasis HLB/ GC-MS	83	Togola and Budzinski, 2007b
		Italy	Po river	Oasis MCX/ HLPC-MS-MS	34	Zuccato *et al.*, 2006
			Lambro river	Oasis MCX/ HLPC-MS-MS	175	
		Portugal	Douro river	Oasis HLB/ LC-MS-MS-ESI	178	Madureira *et al.*, 2009; 2010
		Romania	Somes river	Oasis HLB/ GC-MS	75	Moldovan, 2006
		Serbia	Tamis river	Oasis HLB/ LC-MS-MS	30	Vasiljevi *et al.*, 2009

Table 2.5: (Continuation).

anticonvulsant	CAR	Serbia	Sava river	Oasis HLB/ LC-MS-MS	50	Vasiljevi *et al.*, 2009
			Danube river	Oasis HLB/ LC-MS-MS	130	
		Spain	Ebro river basin	Oasis HLB/ LC-MS	< 60	Gros *et al.*, 2010
			Henares-Jarama-Tajo river	Oasis HLB/ HPLC-MS-ESI	104	Fernández *et al.*, 2010
			Ebro river basin	Oasis HLB/ LC-MS	110	Gros *et al.*, 2006
			Llobregat river	Oasis HLB/ LC-MS-MS	3090	Ginebreda *et al.*, 2010
		Switzerland	Lake Greifensee	Oasis HLB/ GC-MS	< 60	Tixier *et al.*, 2003
			Lake Greifen	Oasis HLB/ GC-MS	60	Öllers *et al.*, 2001
			Aa and Aabach river	Oasis HLB/ GC-MS	250	
		United Kingdom	Ouse river	Oasis HLB/ LC-MS-MS	539	Zhang *et al.*, 2008
		Canada	Ottawa river	Oasis HLB/ LC-ESI-MS-MS	<0.2	Segura *et al.*, 2010
			St. Lawrence river	Oasis HLB/ LC-MS-MS-ESI	2	
			Yamaska river	Oasis HLB/ LC-ESI-MS-MS	4	
			Detroit river	Oasis HLB/ HPLC-MS	40	Hua *et al.*, 2006
		USA	Tennessee river	Oasis HLB/ UPLC	6	Conley *et al.*, 2008
			Loup River	Oasis HLB/ GC-MS	21	Bartelt-Hunt *et al.*, 2009
			Wood River	Oasis HLB/ GC-MS	51	
			Big Blue river	Oasis HLB/ GC-MS	132	
			Salt Creek	Oasis HLB/ GC-MS	296	
analgesic	PAR	France	Lergue river	Oasis MCX, C18, HLB/ GC-MS	72	Togola and Budzinski, 2008
		Serbia	Danube river	Oasis HLB/ LC-MS-MS	78	Vasiljevi *et al.*, 2009
			Tamis river	Oasis HLB/ LC-MS-MS	310	
			Sava river	Oasis HLB/ LC-MS-MS	610	
		Spain	Henares-Jarama-Tajo river	Oasis HLB/ HPLC-MS-ESI	43	Fernández *et al.*, 2010
			Ebro river basin	Oasis HLB/ SPE-LC-MS	80	Gros *et al.*, 2010

Table 2.5: (Continuation).

analgesic	PAR	Spain	Ebro river basin	Oasis HLB/ SPE-LC-MS	250	Gros *et al.*, 2006
			Llobregat river	Oasis HLB/ LC-MS-MS	2420	Ginebreda *et al.*, 2010
		USA	Wood River	Oasis HLB/ GC-MS	35	Bartelt-Hunt *et al.*, 2009
			Salt Creek	Oasis HLB/ GC-MS	39	
			Big Blue river	Oasis HLB/ GC-MS	63	
NSAIDs	IBU	China	Liuxi river	Oasis HLB/ GC-MS	11	Zhao *et al.*, 2010
			Zhujiang river	Oasis HLB/ GC-MS	31	
			Hai river	Oasis HLB/ GC-MS	127	Wang *et al.*, 2010
			Liao river	Oasis HLB/ GC-MS	246	
			Yellow river	Oasis HLB/ GC-MS	416	
			Shijing river	Oasis HLB/ GC-MS	685	Zhao *et al.*, 2010
		Finland	Kokemäenjoki river	Oasis MCX/ HPLC-MS-MS-ESI	64	Lindqvist *et al.*, 2005
		France	Lergue river	Oasis MCX, C18, HLB/ GC-MS	5	Togola and Budzinski, 2008
			Seine estuary	Oasis HLB/ GC-MS	611	Togola and Budzinski, 2007b
			Cortiou rocky inlet	Oasis MCX, C18, HLB/ GC-MS	<1000	Togola and Budzinski, 2008
		Germany	Lake Alster	Oasis HLB/ GC-MS	5	Weigel *et al.*, 2004
			Elbe river	Oasis HLB/ GC-MS	32	
		Italy	Po river	Oasis MCX/ HLPC-MS-MS	17	Zuccato *et al.*, 2006
			Lambro river	Oasis MCX/ HLPC-MS-MS	20	
		Luxembourg	Alzette river	Oasis HLB/ LC-MS-MS	295	Pailler *et al.*, 2009
			Mess river	Oasis HLB/ LC-MS-MS	2383	
		Romania	Somes river	Oasis HLB/ GC-MS	115	Moldovan, 2006
		Spain	Ebro river basin	Oasis HLB/ LC-MS	< 100	Gros *et al.*, 2010
			Cardener river	Oasis HLB/ LC-MS	130	Farré *et al.*, 2001
			Ebro river basin	Oasis HLB/ LC-MS	150	Gros *et al.*, 2006

Table 2.5: (Continuation).

NSAIDs	IBU	Spain	Llobregat river	Oasis HLB/ LC-MS	153	Farré *et al.*, 2001
			Riera Rubi river	Oasis HLB/ LC-MS	1650	
			Anoia river	Oasis HLB/ LC-MS	2700	
			Henares-Jarama-Tajo river	Oasis HLB/ HPLC-MS-ESI	2784	Fernández *et al.*, 2010
			Llobregat river	Oasis HLB/ LC-MS-MS	10000	Ginebreda *et al.*, 2010
		Switzerland	Lake Greifensee	Oasis HLB/ GC-MS	<15	Tixier *et al.*, 2003
			Lake Greifen	Oasis HLB/ GC-MS	15	Öllers *et al.*, 2001
			Aa and Aabach river	Oasis HLB/ GC-MS	80	
		Canada	Detroit river	Oasis HLB/ HPLC-MS	10	Hua *et al.*, 2006
			Halifax watershed	Oasis HLB/ LC-MS	160	Comeau *et al.*, 2008
	DCF	China	Liuxi river	Oasis HLB/ GC-MS	11	Zhao *et al.*, 2010
			Zhujiang river	Oasis HLB/ GC-MS	33	
			Hai river	Oasis HLB/ GC-MS	46	Wang *et al.*, 2010
			Liao river	Oasis HLB/ GC-MS	105	
			Yellow river	Oasis HLB/ GC-MS	136	
			Shijing river	Oasis HLB/ GC-MS	150	Zhao *et al.*, 2010
		Finland	Kokemäenjoki river	Oasis MCX/ HPLC-MS-MS-ESI	35	Lindqvist *et al.*, 2005
		France	Lergue river	Oasis MCX, C18, HLB/ GC-MS	33	Togola and Budzinski, 2008
			Seine estuary	Oasis HLB/ GC-MS	173	Togola and Budzinski, 2007b
			Cortiou rocky inlet	Oasis MCX, C18, HLB/ GC-MS	<1000	Togola and Budzinski, 2008
		Germany	Lake Alster	Oasis HLB/ GC-MS	26	Weigel *et al.*, 2004
			Elbe river	Oasis HLB/ GC-MS	67	
		Luxembourg	Mess river	Oasis HLB/ LC-MS-MS	19	Pailler *et al.*, 2009
			Alzette river	Oasis HLB/ LC-MS-MS	55	

Table 2.5: (Continuation).

NSAIDs	DCF	Spain	Cardener river	Oasis HLB/ LC-MS	51	Farré *et al.*, 2001
			Ebro river basin	Oasis HLB/ LC-MS	< 60	Gros *et al.*, 2010
			Ebro river basin	Oasis HLB/ LC-MS	60	Gros *et al.*, 2006
			Llobregat river	Oasis HLB/ LC-MS	120	Farré *et al.*, 2001
			Henares-Jarama-Tajo river	Oasis HLB/ HPLC-MS-ESI	156	Fernández *et al.*, 2010
			Riera Rubi river	Oasis HLB/ LC-MS	484	Farré *et al.*, 2001
			Anoia river	Oasis HLB/ LC-MS	610	
			Llobregat river	Oasis HLB/ LC-MS-MS	18740	Ginebreda *et al.*, 2010
		Switzerland	Lake Greifensee	Oasis HLB/ GC-MS	<10	Tixier *et al.*, 2003
			Lake Greifen	Oasis HLB/ GC-MS	10	Öllers *et al.*, 2001
			Aa and Aabach river	Oasis HLB/ GC-MS	150	
		United Kingdom	Ouse river	Oasis HLB/ LC-MS-MS	65	Zhang *et al.*, 2008
		Canada	Detroit river	Oasis HLB/ HPLC-MS	15	Hua *et al.*, 2006
	NAP	China	Zhujiang river	Oasis HLB/ GC-MS	5	Zhao *et al.*, 2010
			Yellow river	Oasis HLB/ GC-MS	18	Wang *et al.*, 2010
			Liao river	Oasis HLB/ GC-MS	41	
			Shijing river	Oasis HLB/ GC-MS	125	Zhao *et al.*, 2010
		Finland	Kokemäenjoki river	Oasis MCX/ HPLC-MS-MS-ESI	45	Lindqvist *et al.*, 2005
		France	Lergue river	Oasis MCX, C18, HLB/ GC-MS	9	Togola and Budzinski, 2008
		France	Seine estuary	Oasis HLB/ GC-MS	275	Togola and Budzinski, 2007b
		France	Cortiou rocky inlet	Oasis MCX, C18, HLB/ GC-MS	2000	Togola and Budzinski, 2008
		Spain	Lloregat river	Oasis HLB/ LC-MS	17	Farré *et al.*, 2001
			Ebro river basin	Oasis HLB/ LC-MS	50	Gros *et al.*, 2006
		Spain	Ebro river basin	Oasis HLB/ LC-MS	< 110	Gros *et al.*, 2010
			Anoia river	Oasis HLB/ LC-MS	120	Farré *et al.*, 2001

Table 2.5: (Continuation).

			Cardener river	Oasis HLB/ LC-MS	234	Farré *et al.*, 2001
			Henares-Jarama-Tajo river	Oasis HLB/ HPLC-MS-ESI	640	Fernández *et al.*, 2010
			Riera Rubi river	Oasis HLB/ LC-MS	2000	Farré *et al.*, 2001
			Llobregat river	Oasis HLB/ LC-MS-MS	2060	Ginebreda *et al.*, 2010
		Switzerland	Lake Greifen	Oasis HLB/ GC-MS	10	Öllers *et al.*, 2001
			Lake Greifensee	Oasis HLB/ GC-MS	<15	Tixier *et al.*, 2003
			Aa and Aabach river	Oasis HLB/ GC-MS	400	Öllers *et al.*, 2001
		Canada	Halifax watershed	Oasis HLB/ GC-MS	110	Comeau *et al.*, 2008
anti-lipidemics/ lipid-regulators	GEM	China	Zhujiang river	Oasis HLB/ GC-MS	8	Zhao *et al.*, 2010
			Yellow river	Oasis HLB/ GC-MS	14	Wang *et al.*, 2010
			Shijing river	Oasis HLB/ GC-MS	20	Zhao *et al.*, 2010
			Hai river	Oasis HLB/ GC-MS	87	Wang *et al.*, 2010
		France	Lergue river	Oasis MCX, C18, HLB/ GC-MS	2	Togola and Budzinski, 2008
			Seine estuary	Oasis HLB/ GC-MS	86	Togola and Budzinski, 2007b
		Spain	Ebro river basin	Oasis HLB/ LC-MS	40	Gros *et al.*, 2010
			Ebro river basin	Oasis HLB/ LC-MS	60	Gros *et al.*, 2006
			Cardener river	Oasis HLB/ LC-MS	75	Farré *et al.*, 2001
			Llobregat river	Oasis HLB/ LC-MS	236	
			Anoia river	Oasis HLB/ LC-MS	255	
			Riera Rubi river	Oasis HLB/ LC-MS	457	
			Llobregat river	Oasis HLB/ LC-MS-MS	7780	Ginebreda *et al.*, 2010
anti-lipidemics/ lipid-regulators	GEM	Canada	Halifax watershed	Oasis HLB/ GC-MS	8	Comeau *et al.*, 2008

Table 2.5: (Continuation).

antidepressant	FLX	Spain	Henares-Jarama-Tajo river	Oasis HLB/ HPLC-MS-ESI	66	Fernández *et al.*, 2010
			Ebro river basin	Oasis HLB/ LC-MS	< 110	Gros *et al.*, 2010
			Rubi river	Oasis HLB/ LC-MS	1550	Farré *et al.*, 2001
		USA	Crabtree Creek	C18 Empore disks/ LC-ESI-MS-MS	10	Bringolf *et al.*, 2010
anxiolytic	DIA	Portugal	Douro river	Oasis HLB/ LC-ESI-MS-MS	4	Madureira *et al.*, 2009; 2010
		Romania	Somes river	Oasis HLB/ GC-MS	34	Moldovan, 2006
		Spain	Ebro river basin	Oasis HLB/ LC-MS	< 10	Gros *et al.*, 2010

2.6 Conclusions

Overall, the present study highlights that the use of POCIS devices clearly enabled a suitable quantification and characterization of APIs concentrations in Arade river sites, in which although no clear indication of the summer impact was shown most of the selected APIs were present at relevant concentrations. Nevertheless, for POCIs application potential optimization it is necessary to further define deployment protocol and experimental calibration uniformity in order to enhance the validation and comparability of obtained data. Finally, the high versatility of POCIS on polar and semipolar contaminants quantification in all types of water environments can significantly improve the APIs' ERA in the aquatic system and non-target species going in agreement with WFD implementation intents.

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Chapter 3

Effects of NSAID ibuprofen in mussel *M. galloprovincialis*

Chapter 3A

Gonzalez-Rey, M., Bebianno, M.J., 2011. Non-steroidal anti-inflammatory drug (NSAID) ibuprofen distresses antioxidant defense system in mussel *Mytilus galloprovincialis* gills. *Aquatic Toxicology* 105, 264– 269.

Chapter 3B

Gonzalez-Rey, M., Bebianno, M.J., 2012. Does non-steroidal anti-inflammatory (NSAID) ibuprofen induce antioxidant stress and endocrine disruption in mussel *Mytilus galloprovincialis*? *Environmental Toxicology and Pharmacology* 33, 361-371

3 A. Non-steroidal Anti-Inflammatory Drug (NSAID) Ibuprofen Distresses Antioxidant Defense System in Mussel **Mytilus galloprovincialis** *gills*

Abstract

Active pharmaceutical ingredients (APIs) are presently considered an emergent class of environmental contaminants. Ibuprofen (IBU) is one of the most applied non-steroidal anti-inflammatory drugs (NSAIDs) in the world. Several authors report the occurrence of IBU in influents and effluents of waste water treatment plants (WWTPs), surface, river and public tap water in numerous countries. However, very little is known about the risks and chronic effects of IBU exposure in non-target organisms. This approach undertakes the assessment of several oxidative stress biomarkers responses through the analysis of antioxidant enzyme activities (superoxide dismutase – SOD, catalase – CAT, glutathione S-transferase – GST, glutathione reductase – GR) and lipid peroxidation (LPO) levels in sentinel species mussel *M. galloprovincialis* gills exposed for 2 weeks to an environmental realistic concentration of IBU. Results clearly show the significant induction and positive correlation between SOD activity and LPO in exposed gills, concomitant to an antioxidant defense depletion of CAT, GR and GST compared to controls. The integration of all biomarkers in mussels' gills separates non- and exposed groups supporting the breakdown of the redox defense system and IBU's pro-oxidant action. Further studies are needed to test possible endocrine disruption effects in mussels' reproduction fitness as IBU is involved on prostaglandins biosynthesis inhibition.

3.1 Introduction

One of the highest lifestyle altering improvements in modern societies is unquestionably owed to the application of active pharmaceutical ingredients (APIs) in healthcare practice. The various therapeutic actions attributed to APIs are critical to the treatment and/or prevention of numerous human diseases. However, its high consumption by an exponential worldwide growing population results in its ubiquity in the environment (Togola and Budzinski, 2008). APIs meet all of the emergent pollutant criteria since they (1) occur and induce effects at low concentrations, (2) potential chronic effects at low level exposures are still undefined on ecosystem and human health and/or (3) are after subjected to environmental dispersal after incomplete treatment at WWTP systems. Therefore APIs must be issued on environmental impact studies as stated by EU and US legislation guidelines (Besse and Garric, 2008; Santos *et al.*, 2010). The first report featuring this problematic was published in the late 1970s (Garrison *et al.*, 1976). Since then, the development of new highly sensitive analytic techniques allowed to assess the occurrence, fate and sources of APIs on water bodies (such as waste water treatment plant – WWTPs, tap, surface, ground and marine waters) at concentrations from $ng.L^{-1}$ to $\mu g.L^{-1}$ (see reviews, Kümmerer, 2009; Santos *et al.*, 2010). IBU is a propanoic acid derivative and as most widely used non-steroidal anti-inflammatory drugs (NSAIDs) is a nonselective inhibitor of both cyclooxygenase (COX)-1 and -2 isozymes (Praveen Rao and Knaus, 2008). Pain and/or inflammation triggered by reactive oxygen species (ROS) (e.g. nitric oxide and hydrogen peroxide) promotes COX activities reduction, which in turn results in a decreased of prostaglandins synthesis. This feature confers to IBU its analgesic, antirheumatic and antipyretic therapeutic actions (Praveen Rao and Knaus, 2008). Since, most of the studies concerning exposure to IBU focus on acute (lethal concentration – LC_{50} or maximal effective concentration – EC_{50}) and chronic tests (e.g. reproductive, behavior, growth alterations) (De Lange *et al.*, 2006; Flippin *et al.*, 2007) it is important to address IBU impact on antioxidant system of bivalves as most pharmaceuticals therapeutic action is related to specific redox reactivity (Martín-Díaz *et al.*, 2009). Mussels, as filter-feeders and sessile organisms, are excellent tools on marine and estuarine pollution evaluation being frequently selected to integrate numerous international monitoring programs such as "Global Mussel Watch" (Goldberg *et al.*, 1978).

It is well accepted that aerobic organisms possess regulatory mechanisms which intercept and inactivate toxic ROS induced by contaminants, leveling them to a minimum intracellular stage (Valavanidis *et al.*, 2006). These mechanisms are mediated by antioxidant enzymes (such as: SOD, CAT and glutathione peroxidases (GPx) which increase the synthesis and adapt themselves to stress level fluctuations. If the antioxidant system is overwhelmed, damage in macromolecules occurs, namely LPO which can be translated in phospholipids membrane loss of rigidity and permeably (Valavanidis *et al.*, 2006). Considering the above, this approach undertakes the assessment of several oxidative stress biomarker responses through the analysis of antioxidant enzyme activities (SOD, CAT, GST, GR) and LPO levels in mussel's *M. galloprovincialis* gills exposed for 2 weeks to an environmental realistic concentration of non-steroid anti-inflammatory drug (NSAID) IBU i.e. similar to the ones found in WWTP effluents and surface waters (Thomas and Hilton, 2004; Palmer *et al.*, 2008; Chapter 2).

3.2 Materials and Methods

3.2.1 Chemicals

All the applied chemicals were obtained as described: ibuprofen (2-(4-isobutylphenyl) propanoic acid) (I4883, ≥ 98% GC, CAS: 15687-27-1); 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (D8130, ≥ 98% TLC, CAS: 69-78-3); butylated hydroxytoluene (BHT) (B1378, ≥ 99.0% GC, CAS: 128-37-0); cytochrome *c* from equine heart (C7752, > 95%, CAS: 9007-43-6); diethylenetriaminepentaacetic acid dianhydride (DTPA) (D6148, CAS: 23911-26-4); ethylenediaminetetraacetic acid (EDTA) (ED, ≥ 99%, CAS: 60-00- 4); glutathione reductase (G3664, CAS: 9001-48-3); hydrogen peroxide solution (H1009, 30% w/w, CAS: 7722-84-1); hypoxanthine (H9377, > 99%, CAS: 68-94-0); l-glutathione oxidized (GSSG) (G4501, > 98%, CAS: 27025-41-8); l-glutathione reduced (GSH) (G4251, > 98%, CAS: 70-18-8); xanthine oxidase from bovine milk (X1875, CAS: 9002-17-9); β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADPH) (N8129, ≥ 97%, CAS: 606-68-8); bovine albumin serum (BSA) (A9418, > 98%, CAS: 9048-46-8); 1-methyl-2-phenylindone (99%, CAS: 3558-24- 5); 1.1.3.3. tetramethoxypropane (MDA) (108383, CAS: 102-52-3) were obtained from Sigma–Aldrich (Steinheim, Germany). Bio-Rad protein assay dye reagent concentrate (phosphoric acid CAS: 7664-38-2 + methanol CAS: 67-56-1) was obtained from Bio-

Rad Laboratories, Inc. (USA). Natriumazide (sodium azide) (106688, ≥ 99%, CAS: 26628-22-8); potassium chloride (104936, 99.5%, CAS: 7447-40-7); trichloroacetic acid (TCA) (100807, CAS: 76-03- 9); TRIS (≥ 99%, CAS: 77-86-1); 1,4-dithiothreitol (DTT) (≥ 99%, CAS: 3483-12-3) obtained from Merck (Germany). 1-chloro-2,4,-dinitrobenzene (CDNB) (24440, ≥ 98% GC, CAS: 97-00-7) obtained from Fluka. D(+) saccharose extra pure (16104, 99%, CAS: 57-50-1) obtained from Riedel-de-Haën.

3.2.2 IBU Exposure Assay (250 ng.L^{-1})

Mussels *M. galloprovincialis* (n = 245, overall average shell length size: 69 ± 5 mm, width: 38 ± 3 mm) were collected from a reference site in Ria Formosa Lagoon, Southeast of Portugal, placed in 7 separated aquaria (n = 35, 1 mussel L−1) and acclimated in aerated seawater for 7 days prior to IBU exposure. A 250 ng.L^{-1} concentration of IBU was added on three of these aquaria; the remaining four were used as control. Water was changed every 48-h and IBU concentration re-established. Each aquarium was kept at constant temperature (18 ◦C ± 1), salinity (32.5 ppm ± 1), pH (7.83 ± 0.5) and oxygen saturation (>94%). The treatment took place for 2 weeks; sampling times were set up: 0, 3, 7 and 15 days for control and 3, 7 and 15 days for exposure treatment. Over each selected set of time, mussels (n = 35) were collected from both control and exposure aquaria and subjected to biometric data measurement (shell: length, width). For condition index, mussels' whole body tissue was considered, while for antioxidant enzyme activities and LPO analysis only gills were considered. After dissection, gills were immediately frozen with liquid nitrogen and stored at −80 ◦C until further analysis. Furthermore, the mortality observed was insignificant and no different between treatments (n = 4).

3.2.3 Condition Index

The condition index (CI) was calculated in both non- and exposed mussels (n = 20) over each set of time, by the ratio:

$$CI = \frac{\text{whole soft tissue (wet weight)}}{\text{whole body tissue w/shell (wet weight)}} \text{ x } 100$$

3.2.4 Tissue Preparation for Antioxidant Enzyme Activities Analysis

Previously dissected gills were individually homogenized with 20 mM TRIS buffer (1mM of EDTA, 0.5 M of saccharose, 0.15 M of KCl and 1 mM of DTT), pH 7.6. The homogenates were then, centrifuged at 500 × g for 15 minutes at 4ºC to precipitate cytosolic fraction. The supernatants were recentrifuged for 45 minutes at 12,000 × g at 4ºC; the volumes measured and purified applying Sephadex® G-25 gel columns in order to remove low molecular weight proteins. A 100 µl aliquot was saved for total protein quantification according to Bradford's method (Bradford, 1976) using bovine serum albumin (BSA) as a standard.

3.2.5 Antioxidant Enzyme Analysis

Aliquots of the resulting gills eluted fraction were used for the determination of enzyme activities by spectrophotometry.

SOD activity in mussel gills (n = 5) was evaluated by measuring the reduction of cytochrome *c* absorbance generated by xanthine oxidase/hypoxanthine system at 550 nm (McCord and Fridovich, 1969). The arbitrary units of SOD (U) are expressed as the quantity of enzyme that inhibits cytochrome *c* reduction by 50% per minute of mg^{-1} of total protein.

CAT activity in mussel gills (n = 5) was determined by measuring at 240 nm the absorbance decrease due to hydrogen peroxide (H_2O_2) consumption according to Greenwald (1985). The CAT activity is expressed as $\mu mol.mg^{-1}$ of total $protein.min^{-1}$concentrations.

GR activity in mussel gills (n = 5) was assessed using oxidized glutathione (GSSG) as substrate following Cribb *et al.* (1989) method. The GR activity was measured at 340 nm subsequent to co-factor NADPH oxidation increase and expressed as µmol NADPH oxidized mg $protein^{-1}.min^{-1}$.

GST activity (n = 5) was evaluated with 1-chloro-2,4- dinitrobenzene (CDNB) as a substrate following a modified method of Habig *et al.* (1974). The absorbance values were recorded at 340 nm considering as enzyme activity the µmol CDNB conjugate $formed.mg^{-1}$ of total $protein.min^{-1}$.

3.2.6 LPO Analysis

Dissected gills (n = 10) were homogenized separately with 20 mM TRIS-HCl buffer and butylated hydroxytoluene (BHT) (100:1µl respectively), pH 8.6. The homogenates were then, centrifuged at 30,000 × g for 45 minutes at 4ºC to precipitate cytosolic fraction. An aliquot was saved for total protein quantification according to Bradford's method (Bradford, 1976). An aliquot of the previous cytosolic fraction was also used to determine LPO levels through the quantification of the absorbance of malondialdehyde (MDA) and (2E)-4-hydroxy-2-nonenal (HNE) at 586 nm following an adapted method of Erdelmeier *et al.* (1997). LPO levels are expressed as µmol MDA.g^{-1} protein.

3.3 Statistical Analysis

Two-way ANOVA was performed using STATISTICA® to test treatments (non- and exposure to IBU) and time effect over 2 weeks of conditioning. Duncan's test was applied to discriminate the differences and interactions over time within each biomarker and CI. Pearson correlation was also used to measure the dependency between each biomarker. Principal component analysis (PCA) was used to compare the biomarkers levels responsible for the variance between exposed and non-exposed mussels at each sampling time. PCA was per-formed using XLSTAT® 2010. Statistical significance was defined at $p < 0.05$ level.

3.4 Results

3.4.1 Condition Index

The condition index of mussels is in Table 3.1. Both non-and exposed mussels showed no significant detrimental effects between them on their physiological condition after 2 weeks.

Table 3.1: Condition Index (%) (mean ± standard deviation) of *M. galloprovincialis* non- and exposed to IBU for 2 weeks. Different letters express significant differences ($p < 0.05$).

Time (days)	CI (%)	
	Control	IBU exposure
0	23.7 ± 4.7^{b}	
3	32.0 ± 6.4^{a}	34.7 ± 42^{a}
7	29.6 ± 7.6^{a}	35.1 ± 6.7^{a}
15	22.9 ± 5.7^{b}	23.2 ± 3.8^{b}

3.4.2 Antioxidant Enzymes

The results obtained for SOD activity are in Figure 3.1A. SOD activity was significantly different between treatments being higher in exposed mussels than control on the 3rd (1.4-fold) and 7th (2.7-fold) days. Additionally, considering the variation within treatments, SOD activities in non-exposed mussels did not vary over time ($p > 0.05$) whereas in exposed mussels SOD activity linearly increased (SOD ($U.mg^{-1}$ $protein.min^{-1}$) = 3.6 t (days) + 14.9, $r = 0.998$) over the first week, decreasing to 2.4-fold lower activity by the end of the experiment to an activity not different from the one at the beginning of the experiment. CAT activity (Figure 3.1B) exhibited a totally different behavior from SOD activity. Results show that in both treatments CAT activity decreased over time although more markedly in exposed mussels. The difference between treatments was only significant after day 15, in which CAT activity in exposed mussels' gills was 1.8-fold lower than controls ($p < 0.05$).

Although there was a slight increase in GR activity (Figure 3.1C) in the gills of unexposed mussels this increment was not significant ($p > 0.05$). Remarkably, GR activity is the only antioxidant enzyme consistently inhibited by IBU, since in IBU exposed gills' this enzyme activity significantly decreased after 3 (3.3-fold), 7 (4.4-fold) and 15 (2.8-fold) days compared to each controls ($p < 0.05$).

GST activity (Figure 3.1D) significantly decreased in both non- and IBU exposed organisms, being similar after the first week. How-ever, on the 3rd day of exposure, exposed mussels showed a significant GST activity decrease (about 1.7-fold lower) when compared to controls which in turn presented the highest GST activity (65.2 ± 0.9 µmol CDNB conjugate $formed.min^{-1}.mg\ protein^{-1}$). GST activity was positively correlated with CAT activity ($r = 0.992$, $p < 0.05$), no other correlations were found within antioxidant enzyme activities.

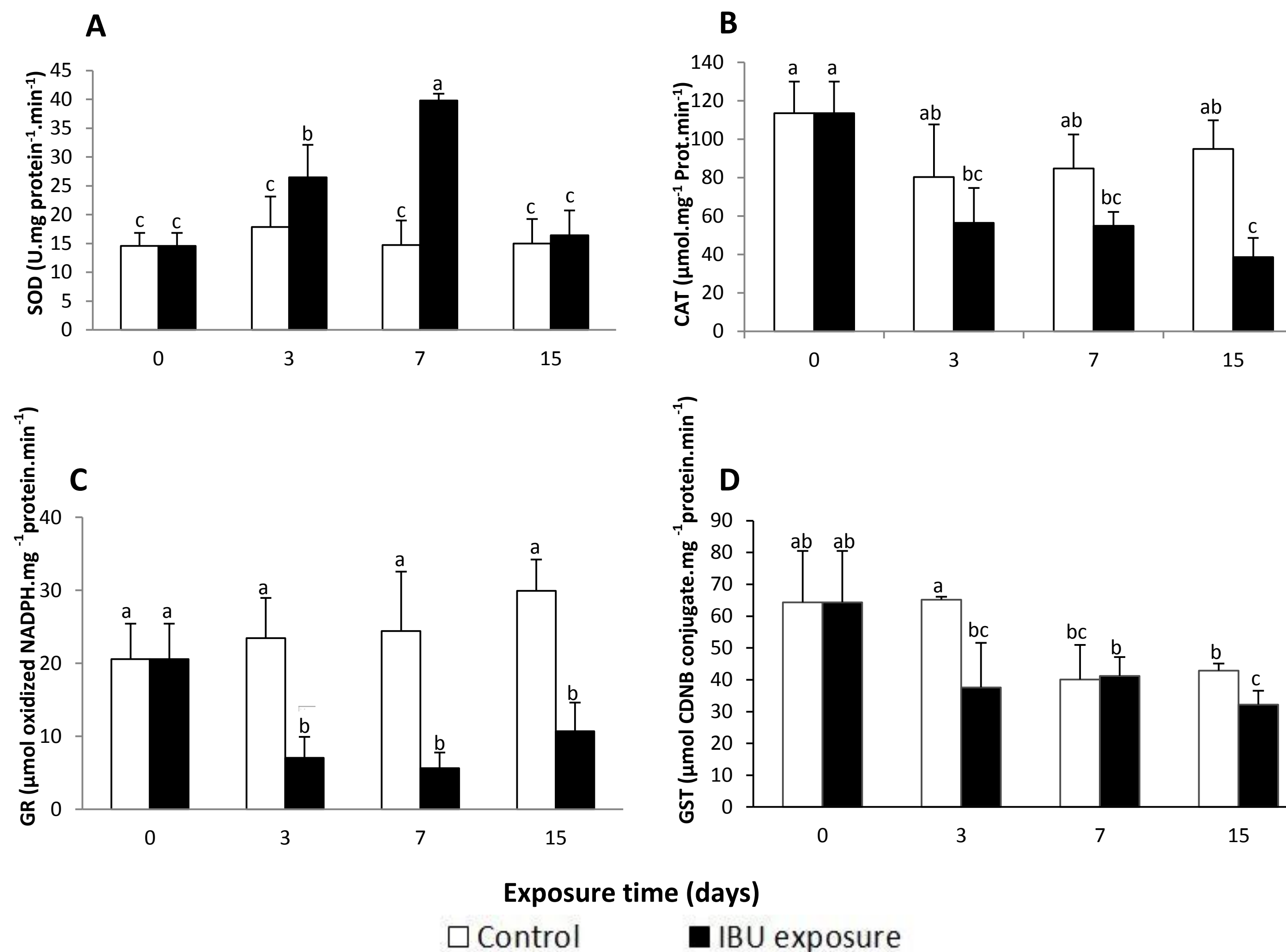

Figure 3.1: Antioxidant enzyme activities (mean ± standard deviation): (A) superoxide dismutase (SOD), (B) catalase (CAT), (C) glutathione reductase (GR) and (D) glutathione S-transferase (GST) in two-week IBU exposed and controls *M. galloprovincialis* gills. Different letters express significant differences ($p < 0.05$).

3.4.3 LPO

Despite LPO levels (Figure 3.2) varied significantly in controls at the beginning and end of the experiment, after 1 week LPO levels were significantly higher (3-fold) in exposed mussels compared to controls ($p < 0.05$), exhibiting a similar linear trend [LPO (µmol MDA.g^{-1} protein) = 2.34 t (days) + 1.27, $r = 0.98$) and direct correlation with SOD activity ($r = 0.971$, $p < 0.05$). Furthermore, from day 7 to 15 LPO significantly decreased (7-fold) in exposed mussels.

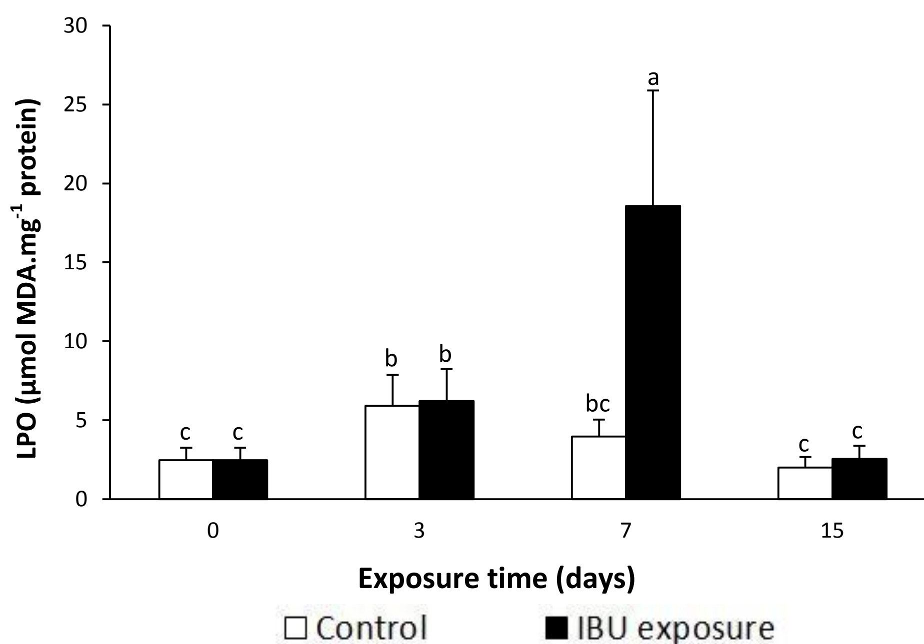

Figure 3.2: Lipid peroxidation (mean ± standard deviation) in two-week IBU exposed and controls *M. galloprovincialis* gills. Different letters express significant differences ($p < 0.05$).

3.4.4 Principal Component Analysis (PCA)

Principal component analysis (PCA) (Figure 3.3) showed that 84.5% of total variance was explained by the two principal components. PC1 expresses 60.4% of total variance, the most significant response refers to the separation between controls, particularly of day 0 and day 7 IBU exposed gills, where the high induction of SOD activity associated to the decrease of the other antioxidant enzyme leads to higher LPO levels. Thereby, mussels' gills exhibit a mirror image response between treatments.PC2 representing 24.2% of total variance, clearly separates day 3 control with day 15 exposed mussels gills, on the later ones all biomarkers and CI significantly decrease. Furthermore within PC2, both day 15 non- and exposed mussels express similar responses, pointing to an antioxidant system recovery.

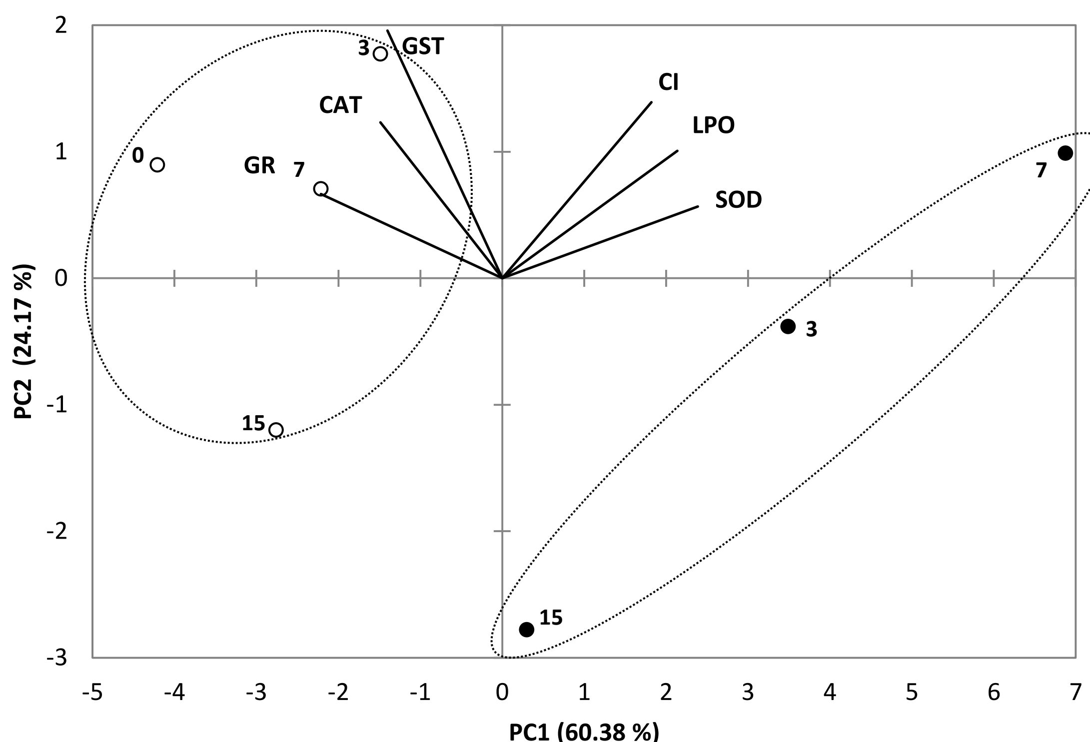

Figure 3.3: PCA corresponding to the integration of antioxidant enzymes SOD, CAT, GR, GST, lipid peroxidation (LPO) and condition index (CI) on total variance (84.5%) between treatments (○– control, ● – IBU exposed mussels) over exposure time (days: 0, 3, 7 and 15).

3.5 Discussion

Our results confirm the alteration of antioxidant enzyme activities in mussels' gills during a short-term exposure to 250 ng.L^{-1} of NSAID IBU, particularly evident by the enhancement of SOD activities over the first week and GR activity overall inhibition in exposed mussels. In order to better interpret an alteration of the antioxidant defense system caused by biologically active contaminants, two aspects have to be taken into account, (1) APIs therapeutic properties are based on redox reactivity and oxidative stability (Harmon *et al.*, 2006; Martín-Díaz *et al.*, 2009) and (2) NSAIDs (such as IBU) are known to disrupt eicosanoid biosynthesis (through the inhibition of COX pathway and prostaglandins) in both invertebrates and vertebrates (Heckmann *et al.*, 2008). The blockage of one of the three membrane-phospholipid arachidonic acid (AA) metabolic pathways (beside, lipoxygenase (LOX) pathway and cytochrome P450 epoxygenase pathway) may distress several physiological functions (such as immune system, reproduction and ion transport) (Gagné *et al.*, 2005; Rowley *et al.*, 2005; Heckmann *et al.*, 2008; Ericson *et al.*, 2010). Furthermore, an increase of ROS is associated to an AA conversion via LOX pathway while the inverse effect can be found when transformed into prostaglandins via COX pathway (Ardaillou *et al.*, 1987). Several studies refer the association of AA accumulation to an enhancement of basal ROS production, hemocytes concentration, phagocytosis and mitochondrial dysfunction either in mammals (Cocco *et al.*, 1999) or in invertebrates (i.e. oysters fed by an AA supplemented diet) (Delaporte *et al.*, 2006). For these reasons, we hypothesize that a possible accumulation of AA in the cells due to COX metabolic pathway obstruction may promote the increment of H_2O_2 production and consequently be other factor contributing for cell oxidative status alteration.

Gagné *et al.* (2006) reports that the exposure to APIs can exhibit detrimental effects on aquatic biota since unlike mammals they are not as efficient eliminating lipophilic drugs and oxygen radicals. Nonetheless, they do possess a detoxification defense system that modulates the increment of intracellular generation of ROS through the alterations of antioxidant enzymatic activities (Viarengo *et al.*, 1995). SOD, CAT and glutathiones are ROS naturally occurring scavengers (Santovito *et al.*, 2005). SOD converts the highly reactive superoxide anion ($O_2^{\cdot-}$) into H_2O_2; which is later degraded to water and oxygen by CAT (Regoli, 1998). In this study, the induction of the detoxifying action of SOD

activity (Figure 3.1A) was triggered over the first week of exposure to IBU followed by a systematic recovery by the end of the experiment. This transitory SOD activity pattern and values were very similar to those found for zebra mussel *Dreissena polymorpha* whole body exposed to 150 $ng.L^{-1}$ of NSAID paracetamol by Parolini *et al.* (2010) even though the timeline refers to the first 24-h, probably associated to the faster size-dependent metabolism of zebra mussel. The consequent elevated generation of H_2O_2 seems not to be counteracted by CAT, GR or GST since these enzymes activities progressively decrease over time; this is further supported by the PCA analysis (Figures 3.1 and 3.3). Relatively to CAT activity (Figure 3.1B) inhibition tendency overtime (significant by the end of the experiment) may be due to an overwhelming excess of H_2O_2 originated as a SOD activity conversion product and the increment of arachidonic acid metabolites (affected by COX pathway blockage). Nonetheless, the concomitant lower activities found in controls suggest other factors affecting mussels' antioxidant system, such as laboratory conditions and absence of food. Martín-Díaz *et al.* (2009) reports no significant alterations on CAT activity in the same mussel species gills when exposed to 100 $ng.L^{-1}$ of anticonvulsant carbamazepine after 7 days. Not directly implicated on the removal of oxygen radicals GR acts as a redox buffer on the maintenance of oxidized glutathione (GSSG)/reduced glutathione (GSH) ratio essential for cell homeostatic status (Vernouillet *et al.*, 2010). In this study GR activity (Figure 3.1C) was the most affected by IBU exposure, being clearly inhibited throughout the duration of the exposure. These results indicate that the presence of IBU alters the gills ability to form reduced glutathione, potentiating the risk of LPO. Consequently, the participation of Phase II detoxifying enzyme GST as a catalyst on the conjugation of GSH to electrophilic centers of several xenobiotics molecules (Regoli, 1998) is compromised, as GST has less substrate to act on hence the significant inhibition after 3 days exposure (Figure 3.1D). As above mentioned it should be taken in account that as CAT, GST activity and mussels' physiologic status given by CI showed similar variation patterns in both treatments highlighting other factors contributing to oxidative status alterations beside the presence of IBU. Nevertheless, LPO levels pattern (Figure 3.2) indicates significant damage in gills membrane structural integrity after 1 week exposure followed by a recovery by the end of the experiment. Moreover, CAT, GST and particularly GR activities inhibition tendency may favor a higher formation of lipid peroxides in the gills. The enhancement of LPO levels after one week are in line with those obtained by Martín-Díaz *et al.* (2009) after exposure to carbamazepine being at

the same time, positively correlated with outlined SOD activity evolution. The integration of all biomarkers in mussels' gills supports the breakdown of the redox defense system and IBU's pro-oxidant action. As shown by PCA (Figure 3.3) after 7 days, there is a clear mirror response between treatments, where the increment of both SOD detoxification response and LPO levels and inverse depletion of CAT, GR and GST activity levels place exposed mussels' gills separately from controls.

3.6 Conclusions

Finally, even considering the contribution of other factors subjacent to the experimental design that should be taken account in the results interpretation, this study reveals that a seemingly low and environmental realistic concentration of NSAID IBU does exert significant fluctuations of several oxidative stress biomarkers in *M. galloprovincialis* gills. The study of other tissues response and the measurement of the uptake of IBU in mussels' tissues would largely complement the obtained data. Overall the application of a multibiomarker approach to assess and highlight short-time effects associated to the presence of these emergent contaminants in sentinel species is further legitimized when considering the plethora of APIs continuously discharged into the aquatic systems and all possible interactions with other pollutants.

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3B. Does non-steroidal anti-inflammatory (NSAID) ibuprofen induce antioxidant stress and endocrine disruption in mussel Mytilus galloprovincialis?

Abstract

Ibuprofen (IBU) is one of the most sold over-the-counter non-steroidal anti-inflammatory drugs (NSAID) and widely detected in the aquatic ecosystems. Nevertheless, the information regarding IBU effects in biota is still sparse. The goal of this study was to assess IBU potential effect as oxidative stress and endocrine disruption inducer in mussel *M. galloprovincialis* applying a battery of biomarkers. Over two weeks of exposure to IBU (250 ng.L^{-1}), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), Phase II glutathione S-transferase (GST) activities and lipid peroxidation (LPO) levels were determined in the digestive gland and alkali-labile phosphates (ALP) were carried out in sex-differentiated mussels' gonads. The results confirm a transitory induction of antioxidant activities responses concomitant to lipid peroxide formation outline and an increase of ALP levels over time, particularly in exposed males which may lead to mussels' reproductive fitness impairment highlighting a higher impact of IBU as an endocrine disruptor than as a short-term reactive oxygen species (ROS)-generator.

3.8 Introduction

Active pharmaceutical ingredients (APIs) are complex bioactive molecules developed to produce an intended therapeutic effect being simultaneous capable to overcome body's natural metabolic degradation (Anderson *et al.*, 2004). These compounds are applied massively both in human and veterinary medicine, reaching the aquatic environment after excretion primarily via waste water treatment plants (WWTPs) effluents, where they are often subjected to an inefficient removal procedure (Ternes, 1998; Metcalfe *et al.*, 2003). Even though APIs are claimed to be the most well-studied chemicals (Van der Ven *et al.*, 2006), their reactivity and associated collateral effects of their usage is still far to be fully understood particularly when considering: (1) the innumerous APIs withdrawn from the markets after years of application and (2) the potential risks for the environment, particularly to non-target organisms. In this context, the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) have established guidelines for the mandatory elaboration of an environmental risk assessment (ERA) in order to authorize any API commercialization (EMEA, 2006; FDA, 1998). Furthermore, based on APIs threshold values of predicted environmental concentrations (PECs) proposed by these agencies, Besse and Garric (2008) developed an APIs exposure priority ranking list from class IA – "highest risk compound" to IV – "very low risk for the environment".

Ibuprofen (IBU) was selected for this study since it is one of the most detected non-steroidal anti-inflammatory drug (NSAID) in the environment worldwide (Table 3.2) and simultaneously defined as a class IA compound by Besse and Garric (2008). In Portugal, IBU was the NSAID most sold over-the-counterdrug in terms of price (~600,000 €), and second after paracetamol in terms of packages number (>168,000) (INFARMED, 2008). NSAIDs are known to promote the non-selective inhibition of cyclooxigenase (COX)-1 and -2 isoforms decreasing the catalysis of prostaglandin (PG) biosynthesis from phospholipid arachidonic acid (AA) (Vane *et al.*, 1998; Fent *et al.*, 2006; Praveen Rao and Knaus, 2008). PGs are responsible for critical physiological functions acting as "local" hormones (Rowley *et al.*, 2005) namely in reproduction, water transport and osmoregulation (Ruggeri and Thoroughgood, 1985; Osada and Nomura, 1990). Accordingly, Fent *et al.* (2006) relate NSAIDs long-term effects as derivative from the alteration of these functions. In humans, prostaglandins inhibition associated to NSAIDs is linked to the reduction of female fertility, miscarriages and

fetal malformations (INFARMED, 2008). In invertebrates, prostaglandins are implicated in the spawning mechanism (Osada and Nomura, 1990). Gagné *et al.*(2005) demonstrated that prostaglandins synthesis and COX activity were inhibited in mussels *Elliptio complanata* after injected with IBU.

Table 3.2: Occurrence of NSAID IBU ($ng.L^{-1}$) in worldwide surface and river water

Country	Environment	IBU (ng.L^{-}	Notes	References
Canada	Detroit river	141	(mean)	Metcalfe *et al.*, 2003
	Hamilton harbour	64	(mean)	
	agricultural surface	30		Lissemore *et al.*, 2006
China	Yellow river	41	(May 2008)	Wang *et al.*, 2010
		11	(December	
	Hai river	75	(July 2008)	
		54	(November	
	Liao river	7	(July 2008)	
		62	(November	
	Pear river	78		Huang *et al.*, 2011
France	surface water	5		Togola and Budzinski,
Germany	river	139		Halling-Sørensen *et al.*,
	Rhine river	41		1998
	surface water	70		Ternes, 1998
	Elbe river	70	(max)	Wiegel *et al.*, 2004
		300	(mean)	Moeder *et al.*, 2000
		32		Weigel *et al.*, 2004b
	Lake Alster	5		
Italy	Po river	4	(1997-2001)	Castiglioni *et al.*, 2004
		7		
		13		Zuccato *et al.*, 2005
	Lambro River	20		
		79		Calamari *et al.*, 2003
Luxembourg	Alzette river	295	(max)	Pailler *et al.,* 2009
	Mess river	2383		
Norway	seawater	8	sum	Weigel *et al.*, 2004b
Poland	surface waters	50	(mean)	Debska *et al.*, 2005
Romania	river	87	(mean)	Moldovan, 2006
South Korea	surface waters	28		Kim *et al.*, 2007
	creek river	100		Yoon *et al.*, 2010
	Han river	23		

Table 3.2: (Continuation).

Spain	rivers	816	(mean of 7 rivers)	Farré *et al.*, 2001
	surface waters	25	(mean)	Gros *et al.*, 2006
Switzerland	river	80		Öllers *et al.*, 2001
	rivers	6		Tixier *et al.*, 2003
UK	Belfast estuary	124		Thomas and Hilton, 2004
	Mersey estuary	250	(average of 6 values)	
	Taff river	20		Kasprzyk-Hordern *et al.*, 2008
	Tees estuary	88		Thomas and Hilton, 2004
	Thames estuary	928		
	Thames river	504	(mean)	Bound and Voulvoulis, 2006
	Tyne estuary	727	(average of 2 values)	Thomas and Hilton, 2004
	Tyne river	597	(average of 18)	Roberts and Thomas, 2006
	stream	1105		Ashton *et al.*, 2004
	small river	1955	(mean)	Bound and Voulvoulis, 2006
USA	streams	200	(mean from 139 streams)	Kolpin *et al.*, 2002
	surface water	180		Cahill *et al.*, 2004
	Mississippi river	34		Zhang *et al.*, 2007
various countries	rivers	152	(max)	Hernando *et al.*, 2006

Bivalves, like mussels, have been successfully applied as bioindicators species, since beside sessile filter-feeders and widely distributed, these organisms have the ability to accumulate the contaminants present in the water throughout their lifespan. This enables the appraisal of biological effects induced by contaminants through the use of biomarkers (Cajaraville *et al.*, 2000; Ortiz-Zarragoitia and Cajaraville, 2006; Porte *et al.*, 2006; Cravo *et al.*, 2009). Several ecotoxicological effects of different APIs have already been evaluated in mussels through the direct and indirect exposure to single and/or mixtures of pharmaceutical ingredients (Table 3.3). However at the present, there is still a lot to unveil concerning the effects of IBU in these organisms: (1) as a potential

oxidative stress inducer since most APIs pharmacodynamics relies on specific redox reactivity (Gagné *et al.*, 2006; Martín-Díaz *et al.*, 2009a) and (2) as a possible endocrine disruptor considering that NSAIDs may alter reproductive functions (Fent *et al.*, 2006). In this perspective, this study comprises a two weeks exposure of mussels *M. galloprovincialis* to an environmental realistic concentration of 250 ng.L^{-1} of IBU in order to better understand the above points: (1) through changes in important mediators of toxic reactive oxygen species (ROS) neutralization in cells like antioxidant enzyme activities: superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), Phase II glutathione S-transferase (GST), and the measurement of lipid peroxides formation by-products (LPO) in digestive gland and (2) through the indirect assessment of vitellogenin-like proteins by alkali-labile phosphates (ALP) method, since their levels are positively correlated (Ortiz-Zarragoitia and Cajaraville, 2006). Vitellogenins precede egg-yolk protein vitellin (Vn) in oviparous species being naturally synthesized in females and inactive in males (Matozzo and Marin, 2008; Matozzo *et al.*, 2008). Whenever elevated levels are detected in males it is a sign of the occurrence of endocrine disruption (ED) (Blaise *et al.*, 1999; Gagné *et al.*, 2002; Matozzo and Marin, 2008; Matozzo *et al.*, 2008). The measurement of ALP released by Vg after alkali hydrolysis is considered a cost-effective ED biomarker in invertebrates (Porte *et al.*, 2006).

Table 3.3: Ecotoxicological effects of APIs in mussel species

Mussel species	Tissue	API concentration	Time	Biological end-points	Reference
zebra mussel *Dreissena polymorpha*	gonads	**fluoxetine** (>100nM) and **norfluoxetine** (> 5 μM)	4h	• ↑ spawning induction	Fong and Molnar, 2008
	hemocytes	**diclofenac** (0.2, 0.5 and 0.8 μM) + **ibuprofen** (0.2, 2 and 4 μM) + **paracetamol** (0.2, 1 and 3 μM)	1h	• high cytogenotoxic potential (paracetamol < diclofenac < ibuprofen)	Parolini *et al.*, 2009
	hemocytes and whole body	**paracetamol** (1, 5, 10 nM)	96 hours	• significant destabilization of the lysosomal membrane (LMS) • ↑ GST ↑ CAT • ↑ SOD (on the first 24 hours at low concentration)	Parolini *et al.*, 2010
mussel *Mytilus galloprovincialis*	digestive gland, gill, mantle/gonad, hemocytes	**carbamazepine** (0.1, and 10 μg.L^{-1})	7 days	• ↑ neutral lipid and lipofuscin accumulation in digestive gland; • ↑ LPO gill and mantle/gonad • ↑ GST and CAT activities in digestive gland and mantle • ↓ 60% and 80% of haemocyte LMS • ↓ cAMP levels and protein kinase A (PKA) activities in all tissues	Martin-Diaz *et al.*, 2009a
	digestive gland and hemocytes	**bezafibrate** and **gemfibrozil** (injection 0.01, 0.1 and 1 nmol/animal)	24 hours	• concentration-dependent lysosomal destabilization and extracellular lysozyme release • ↑ phagocytosis at highest concentration • ↑ glycolytic enzymes phosphofructokinase (PFK), pyruvate kinase (PK), CAT GST, GR and total glutathione • ↓ Palmytoyl CoA oxidase by bezafibrate	Canesi *et al.*, 2007

Table 3.3: (Continuation).

mussel *Mytilus galloprovincialis*	digestive glands and gill	**propanolol** (11 and 147 µg.L^{-1})	10 days	• ↓ feeding rates, ↑ Phase I carboxylesterase (Cbe) in gill, ↓ GST at low concentration, ↑ GST at high concentration,↑ LPO and acetylcholinesterase (AChE) at high concentration; no observed alterations in digestive glands	Solé *et al.*, 2010
		paracetamol (23 and 403 µg.L^{-1})		• ↑ feeding rates, ↑ LPO, ↑ Cbe; CAT and GST not affected digestive glands; ↓ AChE; GST and LPO not affected in gills	
	digestive glands, gills, mantle/gonad	**propanolol** (0.3, 3, 30, 300, 30,000 ng.L^{-1})	7 days	• ↓ cAMP and PKA, ↑ CAT and ↑GST in digestive glands, ↑ cAMP and PKA ↓ CAT at 3 ng.L^{-1} and ↓ CAT > 30 ng.L^{-1} and ↓ GST in the mantle/gonads • unchanged cAMP and PKA in gills concentration-dependent LMS	Franzellitti *et al.*, 2011
freshwater mussel *Elliptio complanata*	digestive gland and gonad	**carbamazepine** (0–10mM)	7 days	• ↑Phase I dibenzylflourescein dealkylase (DBF), ↑ lipid peroxidation (LPO)	Martin-Diaz *et al.*, 2009b
		caffeine (0–10mM)		• ↑DBF, ↑ LPO	
		methotrezate (0–10mM)		• ↑EROD, ↑ DNA damage	
	hemolymph	WWTP effluent	30 days	• ↑ nitric oxide and COX activity • ↑ phagocytosis activity • ↑ ALP [vitellogenin (Vtg-like)]	Bouchard *et al.*, 2009
	gill and gonads	**ibuprofen** (injection 0, 10, 50 and 100 µmol)	24h	• ↓ COX in both tissues and ↑ serotonin transport	Gagné *et al.*, 2005
		WWTP primary-treated effluent		• ↑ COX and state of inflammation	
	visceral mass	**diazepam** (4, 20 and 100 nmol per mussel)	48 hours	• ↓ serotonin and AChE, ↑ dopamine and γ-aminobutyric acid (GABA)	Gagné *et al.*, 2010
		morphine (4, 20 and 100 nmol per mussel)		• ↓ serotonin, ↑ glutamate and dopamine and serotonin-dependent adenylyl cyclase (ADC)	
Baltic Sea blue mussels *Mytilus edulis*	byssus	**diclofenac**, **ibuprofen**, **propranolol** (1, 100, 1000, 5000, 10^5 mg.l^{-1})	19 days	• ↓ scope for growth, ↓ byssus strength, ↓ abundance of byssus threads. • bioaccumulation of diclofenac and propanolol	Ericson *et al.*, 2010

3.9 Materials and Methods

3.9.1 Chemicals

All the applied chemicals were obtained as described: IBU (2-(4-isobutylphenyl) propanoic acid) (I4883, ≥ 98% GC, CAS: 15687-27-1); 1.1.3.3. tetramethoxypropane (MDA) (108383, CAS: 102-52-3); 1-methyl-2-phenylindole (99%, CAS: 3558-24-5); 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) (D8130, ≥ 98% TLC, CAS: 69-78-3); bovine albumin serum (BSA) (A9418 > 98%, CAS: 9048-46-8); butylated hydroxytoluene (BHT) (B1378, ≥ 99.0% GC, CAS: 128-37-0); cytochrome *c* from equine heart (C7752, > 95%, CAS: 9007-43-6); diethylenetriaminepentaacetic acid dianhydride (DTPA) (D6148, CAS: 23911-26-4); ethylenediaminetetraacetic acid (EDTA) (ED, ≥ 99%, CAS: 60-00-4); Fiske and Subbarow Reducer (F5428); glutathione reductase (G3664, CAS: 9001-48-3); HEPES (H3375, >99.5%, CAS: 7365-43-9); hydrogen peroxide solution (H1009, 30%, w/w, CAS: 7722-84-1); hypoxanthine (H9377, >99%, CAS: 68-94-0); L-glutathione oxidized (GSSG) (G4501, > 98%, CAS: 27025-41-8); L-glutathione reduced (GSH) (G4251, > 98%, CAS: 70-18-8); methanesulfonic acid (>99.5%, CAS: 75-75-2); xanthine oxidase from bovine milk (X1875, CAS: 9002-17-9); β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADPH) (N8129, ≥ 97%, CAS: 606-68-8) were acquired from Sigma–Aldrich (Steinheim, Germany). Protein-assay dye reagent concentrate (phosphoric acid CAS: 7664-38-2 + methanol CAS: 67-56-1) was acquired from Bio-Rad Laboratories, Inc. (USA). 1,4-dithiothreitol (DTT) (≥ 99%, CAS: 3483-12-3); acetonitrile (99.8%, CAS: 75-05-8); methanol (99.9%, CAS: 67-56-1); natriumazide (sodium azide) (106688, ≥ 99%, CAS: 26628-22-8); potassium chloride (104936, 99.5%, CAS: 7447-40-7); trichloroacetic acid (TCA) (100807, CAS: 76-03-9); TRIS-(hydroxymethyl)-aminomethane (≥ 99%, CAS:77-86-1) were acquired from Merck (Germany). 1-chloro-2,4,-dinitrobenzene (CDNB) (24440, ≥ 98.0% GC, CAS: 97-00-7); molybdate reagent solution (puriss p.a.) and potassium dihydrogenphosphate (60218, 99.5%, CAS: 7778-77-0) acquired from Fluka. D(+) saccharose extra pure (16104, 99%, CAS: 57-50-1); sodium chloride (NaCl) (puriss p.a., CAS: 7647-14-5); sodium hydroxide (NaOH) (purified, CAS: 1310-73-2) obtained from Riedel-de-Haën.

3.9.2 IBU Exposure Assay (250 ng.L^{-1})

Specimens of mussels *M. galloprovincialis* (n = 150, length size: 69 ± 5 mm, width: 38 ± 3 mm) were collected from Ria Formosa Lagoon, Southeast of Portugal and transported alive to the laboratory in freezing boxes. After shell cleansing, the mussels were acclimated in aerated seawater for one week in 7 separated aquaria. Each aquarium (n = 35, 1 mussel.L^{-1}) was maintained at constant temperature (18º C ± 1), salinity (32.5 ± 1), pH (7.8 ± 0.5) and oxygen saturation (> 94%). A solution of 250 ng.L^{-1} of IBU was prepared in distilled water and added to three of the aquaria; remaining four aquaria used as control. Every two days, seawater was changed and the concentration of IBU restored. The exposure had the duration of two weeks; at days 0, 3, 7 and 15 mussels were sampled from both control and exposure groups and subjected to biometric measurement (shell: length, width). After dissection, digestive glands and sex differentiated gonads were immediately frozen with liquid nitrogen and stored at -80ºC until further analysis.

3.9.3 Tissue Preparation for Antioxidant Enzyme Activities Analysis

Dissected digestive glands were separately homogenized on ice with 20 mM TRIS buffer (1 mM of EDTA, 0.5 M of saccharose, 0.15 M of KCl and 1 mM of DTT) at pH 7.6. The homogenates were centrifuged for 15 minutes at 500×*g* at 4◦C. The obtained supernatants were collected and centrifuged again for 45 minutes at 12,000×*g* at 4◦C. The volume of the supernatants (cytosolic fractions) was measured and 100 µl aliquot collected for the quantification of total proteins. A 2.5 mL aliquot of each cytosolic fraction was purified applying Sephadex® G-25 gel columns to eliminate low molecular weight proteins.

3.9.4 Antioxidant Enzymes Analysis

Aliquots of individual digestive glands purified supernatant were used as triplicates in the quantification of enzyme activities by spectrophotometry.

SOD activity in mussel digestive gland (n = 5) was assessed quantifying 50% of cytochrome *c* absorbance reduction promoted by xanthine oxidase/hypoxanthine system at 550 nm (McCord and Fridovich, 1969).

CAT in mussel digestive gland (n = 5) was evaluated through the decrease of absorbance originated by hydrogen peroxide (H_2O_2) consumption at 240 nm according to Greenwald (1985). The CAT activity is expressed as μmol.mg^{-1}total protein.min^{-1}.

GR activity in mussel digestive gland (n = 5) was assessed according to Cribb *et al.* (1989) method.GR activity was measured at 340 nm through the increase of co-factor NADPH oxidation using as substrate, oxidized glutathione (GSSG), this activity is expressed as μmol NADPH oxidized.mg^{-1} total protein.min^{-1}.

GST activity (n = 5) was estimated according to an adaptation of Habig *et al.* (1974) method.1-chloro-2,4-dinitrobenzene (CDNB) was used as substrate. GST activity was measured at 340 nm and expressed μmol of formed CDNB conjugate.mg^{-1} total protein.min^{-1}.

Total protein concentrations were assessed using bovine serum albumin (BSA) as standard, according to Bradford's method (Bradford, 1976).

3.9.5 LPO Analysis

Digestive glands (n = 10) were individually homogenized on ice with 20 mM TRIS–HCl buffer and butylated hydroxytoluene (BHT) (100:1μL respectively) at pH 8.6. The resulting homogenates were centrifuged for 45 minutes at 30,000 $\times g$ at 4º C in order to obtain the cytosolic fraction. An aliquot was used for the quantification of LPO levels according to Erdelmeier *et al.* (1998) method by the absorbance of malondialdehyde (MDA) and (2E)-4-hydroxy-2-nonenal (4-HNE) at 586 nm and expressed in the total protein content according to Bradford's method (Bradford, 1976) as μmol MDA.g^{-1} total protein.

3.9.6 ALP Analysis

The ALP method was adapted from Gagné and Blaise (1998) and Blaise *et al.* (1999). Sex-differentiated gonads (n = 20) were homogenized in ice with 25 mM Hepes–NaOH buffer (125 mM NaCl, 1 mM dithiothreitol (DTT) and 1 mM EDTA) at pH 7.4. The homogenates were centrifuged at 12,000$\times g$ at 2ºC for 30 minutes; the resulting supernatant (cytosolic fraction) was separated from the pellet. A supernatant aliquot was reserved for total protein content, another adjusted with 35% acetone and re-centrifuged

at 10,000×*g* for 5 min. The resulting liquid phase was discarded and the pellet dissolved with 1 M NaOH in a heating bath at 60ºC for 30 minutes. The concentration of inorganic phosphate (KH_2PO_4) was assessed by phosphomolybdenum method according to Stanton (1968) at 660 nm. Total ALP concentrations are expressed as µg $[PO_4].mg^{-1}$ total protein.

3.9.7 Statistical Analysis

All results are presented as mean ± standard deviation according to each selected sample time (0, 3, 7 and 15 days). In order to verify significant differences in the biomarker responses between and within non- and exposed individuals, parametric one-way analysis of variance (ANOVA) was applied. If the parametric assumptions (normality and homogeneity) were not met non-parametric Kruskal–Wallis test was used. When necessary, Tukey's, Dunn's or Duncan's tests were used to discriminate significant mean differences. Pearson correlation was applied to test significant correlations dependency between each biomarker (Zar, 1999). These analyses were performed using SigmaPlot 11.0®.

Principal Component Analysis (PCA) was used to evaluate variable accountability for the variance between exposed and non-exposed mussels at each sampling time. PCA was performed using XLSTAT® 2010. Statistical significance was set at $p < 0.05$.

3.10 Results

3.10.1 Antioxidant Enzymes

SOD activity in the digestive gland (Figure 3.4A) showed no significant differences between unexposed mussels ($p > 0.05$). In contrast, SOD activity in exposed digestive gland increased over the course of the first week reaching on day 7 an activity 1.7-fold higher than in controls. This was followed by a steep decrease (about 1.5-fold lower than in the previous week) by the end of the exposure reaching a value similar to controls. CAT activity in the digestive gland (Figure 3.4B) like SOD activity showed no significant differences between controls ($p > 0.05$). In exposed individuals, digestive gland CAT activity was about 2-fold higher on the 3rd and 7th day when compared to controls ($p < 0.05$), decreasing to control levels after two weeks ($p > 0.05$).

GR activity (Figure 3.4C) exhibited a very akin pattern to SOD activity, showing also no significant differences between controls ($p > 0.05$). Over the first week of exposure, GR activity increased linearly (GR (μmol oxidized NADPH.mg^{-1} protein.min^{-1}) = 1.5t (days) + 6.7, $r = 0.99$), reaching 2.8-fold higher activity than controls ($p < 0.05$). After two weeks of exposure GR activity decreased (about 2.4-fold), reaching a value not different from controls.

GST activity (Figure 3.4D) unlike any other antioxidant enzymes exhibited no significant differences between either controls or IBU-exposed mussels' digestive gland. Although there was a slight increase ($p > 0.05$) in GST activity in the digestive gland of exposed mussels over time this increase was not significantly different from unexposed mussels at each set of time. Furthermore, GR and CAT activities were positively related ($r = 0.966$, $p < 0.05$).

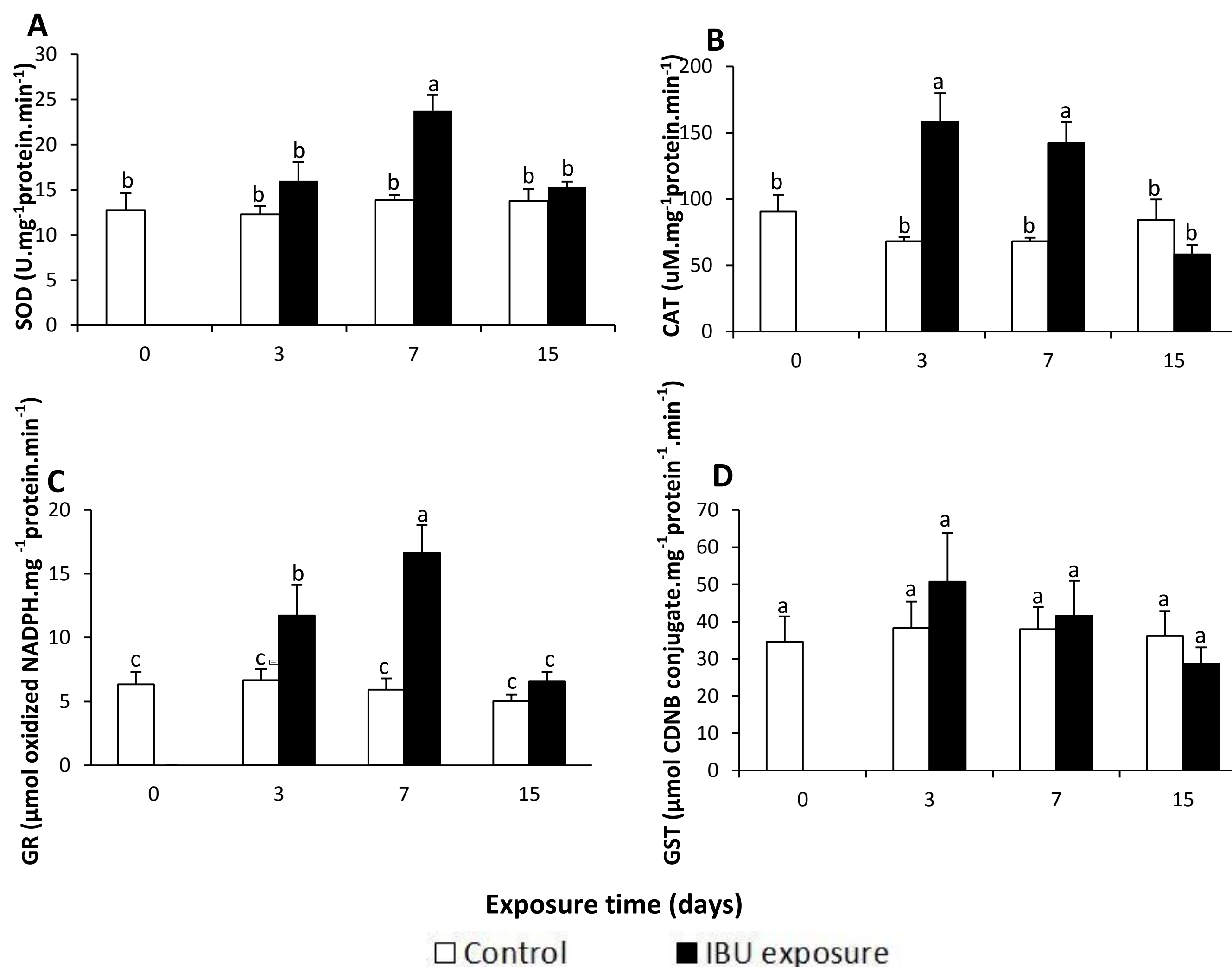

Figure 3.4: Antioxidant enzyme activities (mean ± standard deviation): (A) superoxide dismutase (SOD), (B) catalase (CAT), (C) glutathione reductase (GR) and (D) glutathione S-transferase (GST) in *M. galloprovincialis* digestive gland non- and exposed to IBU for 15 days. Different letters bars are significantly different ($p < 0.05$).

3.10.2 LPO

In unexposed mussels, lipid peroxidation levels (Figure 3.5) was 1.6-fold higher from days 0 to 3 followed by a 1.9-fold decrease from days 7 to 15 reaching levels similar to the beginning of the experiment ($p < 0.05$). Exposed mussels exhibited a similar induction pattern to SOD and GR activities on the first week, where LPO levels increased linearly (LPO (µmol MDA. g^{-1} protein) = 0.5t (days) + 1.5, $r = 0.93$) reaching 2.4-fold higher levels than controls ($p < 0.05$). This was followed by a significant decrease (about 3.8-fold lower) by the end of the two weeks. Additionally, LPO levels were directly related with GR activity ($r = 0.975$, $p < 0.05$).

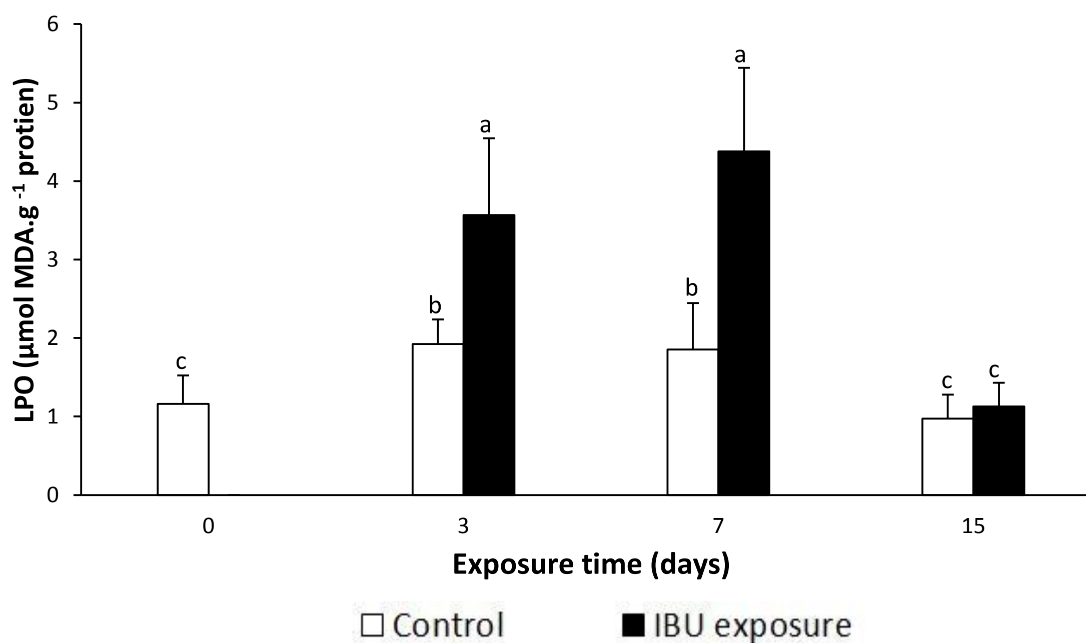

Figure 3.5: Lipid peroxidation (LPO) (mean ± standard deviation) in control and IBU exposed digestive gland of mussel *M. galloprovincialis* over 15 days. Different letters bars are significantly different (*p* < 0.05).

3.10.3 PCA – Oxidative Stress Biomarkers

The PCA (Figure 3.6) integrating the results of antioxidant enzymatic activities and LPO between treatments expressed 94.3% of total variance in digestive gland. PC1 contributes with 72% of total variance, separating clearly Phase II enzyme GST detoxifying activity from other antioxidant enzyme activities and LPO levels. In this sense, all controls and exposed digestive gland from day 15 show very similar responses, although controls at the beginning of the experiment exhibit further influence of GST activity. Oppositely, both exposed digestive glands from days 3 and 7 express their dependency on all the other biomarkers, with responses markedly divergent to the ones of controls. PC2 represents 22% of the total variance, revealing that GST activity is responsible for the difference between early days and controls at the end of the experiment. Simultaneously, day 3 exposed digestive gland variance is due to the increase of CAT activity and LPO levels, while digestive gland exposed for a week is more affected by SOD and GR activities. On the overall, it is evident that particularly after one week of exposure, all antioxidant biomarkers with the exception of GST were significantly induced by IBU. These pro-oxidant responses were also followed by a clear recovery of the antioxidant enzymatic system with reduction of lipid damage at the end of the experiment not different from controls further corroborated by day 15 treatments overlapping response.

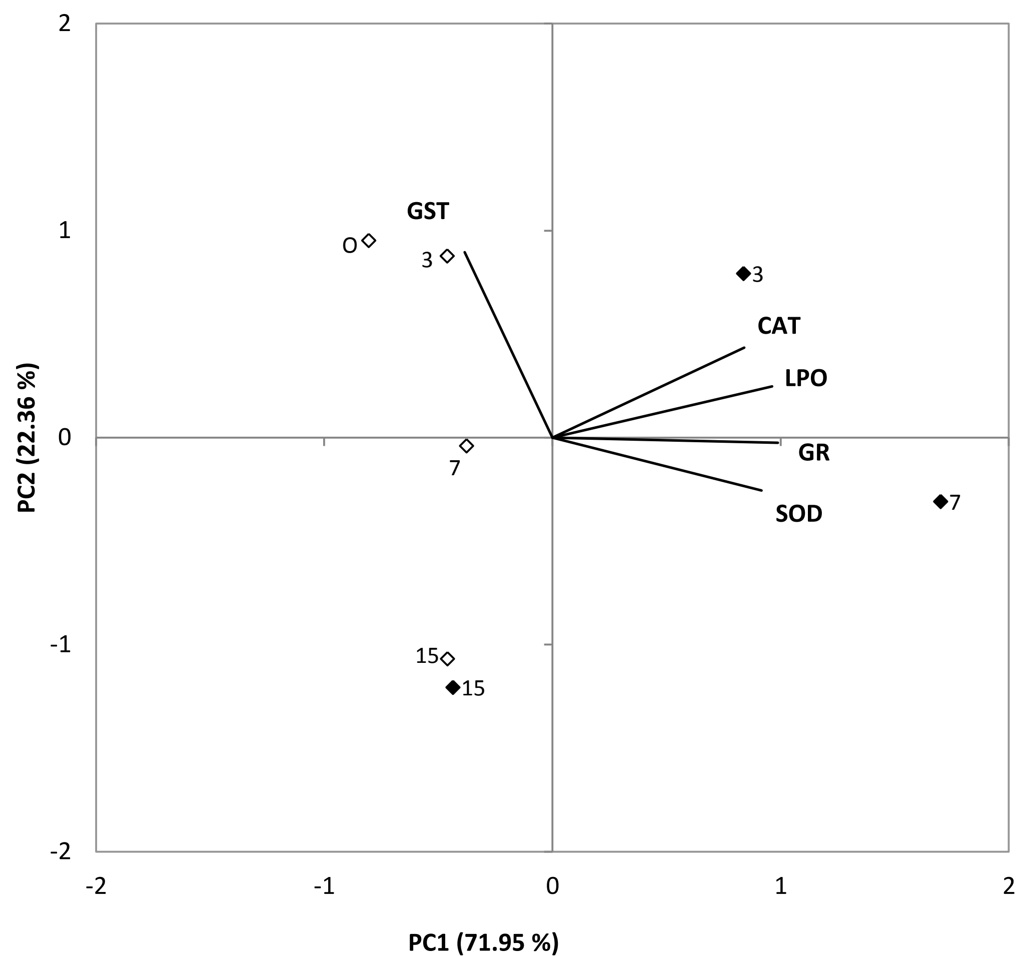

Figure 3.6: Principal component analysis (PCA) integrating antioxidant enzymes (SOD, CAT, GR, and GST) and LPO on total variance (94.3%) between treatments (◊– control, ♦ – IBU exposed mussels) over exposure time (days: 0, 3, 7 and 15).

3.10.4 ALP

ALP levels (Table 3.4) in female and male mussels' gonads controls were very similar over the first week ($p > 0.05$), after which males exhibited a significant lower value than females, and inversely by the end of the second week females showed a significantly lower value than males. On the 3rd day of IBU exposure there was a significantly different gender specific response, in fact exposed males showed higher levels of ALP than exposed females ($p < 0.05$), this tendency continued until the end of the experiment only not significant by day 7 ($p > 0.05$). Furthermore, exposed female gonad showed a linear increase of ALP ((µg $[PO_4]$.mg^{-1} total protein) = 5.3t (days) + 46, $r = 0.98$) through the first week, followed by a slight but not significant concentration decrease. Overall, both IBU exposed males and females (except for day 3) exhibit significantly higher levels of ALP than the respective-day control, ranging from ♂34–117 and ♀39–85 µg $[PO_4]$.mg^{-1} total protein.

Table 3.4: ALP concentrations in sex-differentiated *M. galloprovincialis* gonads exposed to 250 ng.L^{-1} of IBU for two weeks

Treatment	day	ALP concentration (µg PO_4.mg^{-1} total protein)	
		Females	Males
control	0	48.3 ± 8^{cde}	46.3 ± 13^{cde}
	3	41.7 ± 11^{cde}	47.1 ± 14^{cde}
	7	54.5 ± 5^{cd}	34.1 ± 10^{e}
	15	38.6 ± 7de	48.2 ± 8^{cde}
IBU exposure	3	59.5 ± 8^{c}	88.9 ± 23^{b}
	7	85.3 ± 11^{b}	89.6 ± 6^{b}
	15	78.4 ± 19^{b}	116.9 ± 35^{a}

Note: Different letters signal significant differences between and within non- and exposed gonads ($p < 0.05$)

3.11 Discussion

WWTPs continuously receive urban and hospital effluents that include several concentrations of APIs, for which conventional removal treatment technologies have not been specifically designed (Carballa *et al.*, 2004; Reif *et al.*, 2008). Although some APIs may have low persistence, the constant load may dominate over the transformation rate in the WWTPs (Bendz *et al.*, 2005), furthermore polar molecules with low adsorption coefficients like anti-inflammatory drugs tend to remain in the aqueous phase favoring their mobility and reaching the aquatic environment (Carballa *et al.*, 2004). Before being excreted via urine and faeces, APIs like IBU are partially metabolized to Phase I or Phase II metabolites in the human body (Halling-Sørensen *et al.*, 1998; Daughton and Ternes,1999). The elimination rates vary depending on the individual, drug and dosage (Bound and Voulvoulis, 2004). Phase I reactions result in the addition of a reactive functional group to the molecule and often converting it into a more toxic compound either by parental compound oxidation, reduction or hydrolysis. Whereas Phase II or conjugation reactions promote the binding of functional groups to the parental compound through i.e. glutathione conjugation, enhancing excretion by its transformation to more hydrophilic metabolites (Halling-Sørensen *et al.*, 1998; Daughton and Ternes, 1999).

3.11.1 Oxidative Stress

Even though mussels are able to use Phase I or Phase II enzymes to biotransform xenobiotic organic chemicals, they are not as efficient as vertebrates (Gagné *et al.*, 2007). In this sense, the antioxidant defense system is crucial in the neutralization of generated ROS by APIs redox reactivity (Viarengo *et al.*, 1995; Regoli *et al.*, 2002a, b). This system is mediated by a cascade of antioxidant enzymes which scavenge ROS to less toxic compounds. SOD leads this mechanism converting the highly reactive superoxide anion ($O_2^{\cdot-}$) into H_2O_2 (Viarengo *et al.*, 1995). In IBU-exposed mussels' digestive gland SOD activity is enhanced significantly over the first week (Figure 3.4A), the activation of this antioxidant enzyme favors the evidence of a pro-oxidant action of IBU which at the 3rd day is firstly offset by the induction of CAT activity action (Figure 3.4B). As a catalyst of SOD activity by-product H_2O_2 to form water and oxygen, CAT action is enabled by the slight enhancement of SOD activity over the first

days ($p > 0.05$) and/or by the possible increment of H_2O_2 formation in the cells due to the increase of ROS linked to arachidonic acid metabolism via lipoxygenase (LOX) pathway instead of COX pathway blockage by NSAIDs (Ardaillou *et al.*, 1987). There are few studies concerning SOD activity alterations related to APIs exposure, however similar values were reported for zebra mussel *D. polymorpha* whole body exposed to 150 ng.L^{-1} of NSAID paracetamol after 24 hours of exposure which progressively decrease to control levels over 96 hours, whereas CAT activity induction is slower and less pronounced (Parolini *et al.*, 2010). Moreover, the CAT activity induction was also observed for *M. galloprovincialis* digestive gland 24 hours after injection with 0.1 nmol/animal of blood lipid lowering pharmaceuticals (bezafibrate and gemfibrozil) (Canesi *et al.*, 2007) and after one week of exposure to 100 ng.L^{-1} of anticonvulsant carbamazepine and 3 ng.L^{-1} DL-propranolol (Martín-Díaz *et al.*, 2009a; Franzellitti *et al.*, 2011). As shown by PCA (Figure 3.6), exposed digestive gland show a primarily induction of CAT activity at day 3, whereas SOD activity is more preponderant after one week of IBU exposure. The same occurred for GR ($p < 0.05$) (Figures 1C and 3), which indicates the necessity of oxidized glutathione (GSSG) conversion to reduced glutathione (GSH). This resulting GSH can act both as a direct ROS scavenger and/or as a cofactor of several antioxidant enzymes (Regoli *et al.*, 2002b). In these terms, since GST activity outline (Figure 3.4D) is directly related to CAT activity pattern there are evidences that part of the GSH may have been used (1) as substrate by Phase II GST in exposed digestive gland promoting the conjugation reaction with either parental IBU or its metabolites, and/or (2) used to directly neutralize ROS action. Other authors also verified a simultaneous enhancement of CAT and GST activity in *M. galloprovincialis* exposed to bezafibrate (Canesi *et al.*, 2007), carbamazepine (Martín-Díaz *et al.*, 2009a) and DL-propranolol (Franzellitti *et al.*, 2011).

In mussels *M. galloprovincialis* gills exposed to the same IBU concentration, except for the 2-fold higher SOD activity, all antioxidant enzymes were significantly inhibited over time (Gonzalez-Rey and Bebianno, 2011). In fact, this may be linked to specific tissue functions in mussels. Gills are the first organ capable for a strong initial antioxidant response, however unable to counteract further ROS induction. While in digestive gland tissues, the concomitant increment of all antioxidant enzymes justifies its role as the organ primarily responsible for the detoxification of IBU-induced ROS and homeostasis status recovery.

Moreover, lipid peroxidation levels (LPO) were significantly enhanced by IBU presence over the first week ($p < 0.05$) (Figure 3.5), indicating damage derived from oxidative stress. The induction of LPO levels was also reported for digestive gland of *M. galloprovincialis* exposed to 23 $\mu g.L^{-1}$ of paracetamol (Solé *et al.*, 2010) and of *E. complanata* exposed to 0.4, 2 and 10 mM carbamazepine (Martín-Díaz *et al.*, 2009b) (Table 3.2). Interestingly, the direct relationship between LPO levels and GR activity suggests that the induced formation of GSH was not enough to neutralize the presence of oxyradicals throughout the first week of exposure. However, as it can be further corroborated by the overlap between non- and exposed digestive glands on day 15 (Figure 3.6), after IBU pro-oxidant action, the concomitant antioxidant enzyme activities increment favors the recovery to homeostatic baseline levels.

3.11.2 Endocrine Disruption

To our knowledge this is the first study using ALP method in gonads to test a potential effect of endocrine disruption caused by the exposure to an environmental realistic IBU concentration in mussels. The quantification of ALP levels is an effective indirect assessment of xenoestrogenicity in bivalves (Blaise *et al.*, 1999; Gagné *et al.*, 2001; 2002; Matozzo and Marin, 2008). As referred above, inhibition in COX activity in freshwater mussels' *E. complanata* was observed after injection with 100 µM of IBU. However when *E. complanata* were exposed for 30 days to a primary-treated municipal extract containing IBU and other NSAIDs in the range of $\mu g.L^{-1}$, this was not sufficient to inhibit COX activity mussels and rather shown increased spawning and Vg-like proteins content (Gagné *et al.*, 2005). In fact in the present study, ALP levels were significantly enhanced in both exposed males and females' gonads at every selected time of exposure comparing to controls ($p < 0.05$) (Table 3.4), particularly in exposed males where after one week ALP levels were 2-fold higher than controls. Gagné *et al.* (2011) associated the induction of Vtg-like proteins as a biomarker of feminization in mussel *E. complanata*, through the assessment of female/male ratio and the expression of a female-specific protein in males. Even though, the present results are strictly related to ALP level alterations, evidences of serious interferences in male gonads were clearly elicited by IBU. This can potentially lead to the impairment of their reproductive fitness or ultimately its feminization.

3.12 Conclusions

In conclusion, the exposure to an environmental realistic concentration of IBU (250 $ng.L^{-1}$) induces significant transitory antioxidant defense responses and membrane damage in the digestive gland of mussel *M. galloprovincialis*. In addition, IBU exposure results give evidence to endocrine disruption effects in mussels' gonads which may debilitate particularly males' reproductive health. The present study highlights the higher and more progressive impact of IBU as an endocrine disruptor than as a short-term ROS-generator. Further studies are needed to assess and underline chronic and short-term effects in mussels associated to the multiplicity of therapeutic classes of APIs present in the aquatic environment.

3.13 References

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Chapter 4

Effects of SSRI fluoxetine in mussel *M. galloprovincialis*

Gonzalez-Rey, M., Bebianno, M.J., 2013. Does selective serotonin reuptake inhibitor (SSRI) fluoxetine affect mussel *Mytilus galloprovincialis*? *Environmental Pollution* 174, 200-209. doi:10.1016/j.envpol.2012.10.018

4 *Does Selective Serotonin Reuptake Inhibitor (SSRI) Fluoxetine Affect Mussel* Mytilus galloprovincialis*?*

Abstract

Fluoxetine (FLX) the active pharmaceutical ingredient (API) in Prozac® is a widely prescribed psychoactive drug which ubiquitous occurrence in the aquatic environment is associated to a poor removal rate in waste-water treatment plant (WWTP) systems. This API acts as a selective serotonin reuptake inhibitor (SSRI) frequently reported to cause disrupting effects in non-target species. The objective of this study includes a multibiomarker response evaluation on mussel *M. galloprovincialis* during two weeks exposure to 75 ng.L^{-1} FLX assessing antioxidant enzyme activities - superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST); lipid peroxidation (LPO), acetylcholinesterase (AChE) neurotoxic response and endocrine disruption through alkali-labile phosphates (ALP) indirect measurement of vitellogenin-like proteins. Results show transient tissue-specific enzymatic responses and damage affecting mostly mussel gills. However, the clear ALP levels inhibition throughout time in both sex-differentiated gonads gives evidence to FLX reinforced action as an endocrine disruptor rather than an oxidative or neurotoxic inducer.

4.1 Introduction

Ecotoxicological risks associated to the ubiquitous occurrence of active pharmaceutical ingredients (APIs) in aquatic ecosystems are far from known. As the detection technology improves, a larger variety of APIs are being detected in the aquatic environment (see reviews: Calisto and Esteves, 2009; Kümmerer, 2009; Li and Randak, 2009; Alonso *et al.*, 2010; Pal *et al.*, 2010; Kümmerer, 2010; Santos *et al.*, 2010, Brausch and Rand, 2011). Waste water treatment plants (WWTPs) are still ill-equipped for the constant APIs discharge load removal, consequently these bioactive compounds end up entering surface waters (rivers, estuaries and lakes) particularly via household and hospital treated effluents, and from there often recycled to drinking water (Ternes, 2001; Ternes *et al.*, 2002; Stackelberg *et al.*, 2004; Jones *et al.*, 2005; Gibs *et al.*, 2007; Kim *et al.*, 2007; Daughton, 2010) posing potential risks to non-target aquatic life (Fong, 2001; Brooks *et al.*, 2005; Fent *et al.*, 2006).

Fluoxetine (FLX) present in antidepressant Prozac® the most widely prescribed psychoactive drug in the market and like others (e.g. citalopram, fluvoxamine, paroxetine, and sertraline) act as selective serotonin reuptake inhibitor (SSRI) in the treatment of depression, and other mood disorders by increasing the serotonin (5-HT) levels in neuron synaptic space (Brosen, 1993; DeVane, 1999; Hiemke and Härtter, 2000; Fent *et al.,* 2006). Even though, FLX is excreted via urine approximately 10-30% as unchanged parent compound or metabolized to norfluoxetine (DeVane, 1999; Hiemke and Härtter, 2000; Fong and Molnar, 2008) is resilient to hydrolysis, photolysis and microbial degradation processes (Kwon and Armbrust, 2006) occurring in the aquatic environment at $ng.L^{-1}$ (Table 4.1).

Table 4.1: Concentrations of FLX in the aquatic environment

Country	Concentration ($ng.L^{-1}$)	Environment	Reference
Canada	46	surface water	Metcalfe *et al.*, 2003
	99	WWTP effluent	
	509	WWTP effluent	Chen *et al.*, 2006
Croatia	66	surface water	Gros *et al.*, 2006
South Korea	2 – 7	river water and creek	Yoon *et al.*, 2010
	2	WWTP effluent	Trenholm *et al.*, 2006; Kim *et al.*, 2007
Spain	18-66	river water	Fernández *et al.*, 2010
	100	river water	Gros *et al.*, 2010
	8-44	WWTP downstream	Alonso *et al.*, 2010
	19-299	WWTP effluent	Martínez *et al.*, 2007
	70	WWTP effluent	Gros *et al.*, 2006
Sweden	225	WWTP effluent	Zorita *et al.*, 2007
USA	5	reclaimed water facility effluent	Kinney *et al.*, 2006
	12-20	stream	Schultz and Furlong, 2008
	3	surface water	Vanderford and Snyder, 2006
	12	surface water	Kolpin *et al.*, 2002
	111	surface water	Bringolf *et al.*, 2010
	14	water	Stackelberg *et al.*, 2007
	17-25	WWTP effluent	Vanderford and Snyder, 2006
	18	WWTP effluent	Stackelberg *et al.*, 2004
	21	WWTP effluent	Glassmeyer *et al.*, 2005
	58	WWTP effluent	Schultz and Furlong, 2008
	540	WWTP effluent	Weston *et al.*, 2001
	560	WWTP effluent	Benotti and Brownawell, 2007

Since SSRIs alter neurotransmitter 5-HT regulation, which has been associated to the modulation of important functions in hormonal and neuronal mechanisms in both vertebrates and invertebrates (Fong, 2001; Fent *et al.*, 2006; Stanley, 2007; Painter *et al.*, 2009; Styrishave *et al.*, 2011) most peer reviews on FLX exposure ecotoxicological effects focus on acute FLX toxicity and/or physiological, behavioral (mobility, feeding habits and aggression) and reproductive fitness alterations (Table 5.2). Nevertheless, FLX has also been related to affect antioxidant system in mice (Djordjevic *et al.*, 2011). Oxidative stress is characterized by the imbalance when xenobiotic-mediated enhancement of reactive oxygen species (ROS) (e.g. superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot -}$)) exceed exposed aerobic organism's antioxidant defense mechanisms. One of these mechanisms involves the counteracting response of antioxidant enzyme activities such as, superoxide dismutase (SOD) and catalase (CAT) and glutathiones (peroxidase - GPx and reductase - GR) (Livingstone, 2001; Regoli *et al.*, 2002a, b; Valavanidis *et al.*, 2006). Additionally, Phase II glutathione S-transferase enzyme also enables detoxification, acting as a catalyst in conjugation reactions between glutathione with xenobiotic compounds electrophilic centers (Regoli and Principato, 1995). When the antioxidant system response is compromised by an ROS excess, lipid peroxidation (LPO) occurs, resulting in the damage of phospholipids membrane (Valavanidis *et al.*, 2006). The fluctuation of antioxidant enzymes parallels to lipid peroxidation generation due to contaminant exposure have been used successfully as oxidative stress and damage biomarkers in mussel species (Regoli and Principato, 1995; Regoli *et al.*, 2002a; Santovito *et al.*, 2005; Bebianno *et al.*, 2005).

Table 4.2: FLX effects in aquatic molluscs and fish

Species Name	Exposure concentration	Exposure time	Test	Biological end-points	Reference
***Mytilus edulis* (common mussel)**	0.15 mg.L^{-1}		reproduction	• Induction of spawning	Hazardous Substances Databank (HSDB), 2005
***Sphaerium striatum* (fingernail clam)**	5 µM FLX + 5 – 500 µM 5-HT	3 hours	reproduction	• Induction of parturition	Fong *et al.*, 1998
***Dreissena polymorpha* (zebra mussel)**	5 x 10^{-7} from 5 x10^{-4} M	1 hours	reproduction	• Induction of spawning in males	Fong, 1998
	5 x 10^{-6} from 5 x10^{-5} M	2 hours		• Induction of spawning in females	
	>100nM	4 hours		• Induction of spawning and parturition	Fong and Molnar, 2008
	20 – 200 ng.L^{-1}	6 days		• Induction of spawining • Dose dependent decrease oocytes (40-70%) and spertatozoan (21-25%)	Lazzara *et al.,* 2012
***Elliptio complanata* (freshwater mussel)**	300 µg.L^{-1}	96 hours	reproduction	• Induction of parturition of nonviable larvae from female	Bringolf *et al.*, 2010
	3000 µg.L^{-1}	48 hours		• Induction of spermatozeugmata release	
	caged freshwater mussels at stream sites near a municipal WWTP		bioaccumulation	• 79.1 ng.g^{-1} ww (bioaccumulation)	
	injection 2.5 µg		metabolism	• Increase of 5-HT response factor	Gagné and Blaise, 2003
***Anodonta cygnea* (freshwater mussel)**	1×10^{-6} M + presence of light	24 hours	reproduction	• Induction of parturition	Cunha and Machado, 2001
Potamopyrgus antipodarum (NewZealand mudsnail)	3.7, 11.1 and 100 µg.L^{-1}	42 days	reproduction	• Decrease of neonates number per living adult at 100 µg.L^{-1} FLX • Increase of shelled embryos and embryos total number at 3.7 and 11.1 µg.L^{-1} FLX • Increase of unshelled embryos number at 3.7 µg.L^{-1} and decrease at 100 µg.L^{-1}) • Thinner gonad tissues at 100 µg.L^{-1}	Gust *et al.*, 2009
	69 µg.L^{-1}		reproduction	• Decrease of reproduction (number of newborns per individual)	Péry *et al.*, 2008

Table 4.2: (Continuation).

Potamopyrgus antipodarum (NewZealand mudsnail)	0.64, 3.2, 16, 80, and 400 μg.L^{-1}	14-56 days	behavior and reproduction	• Reduction of the mean embryo number at 80 and 400 μg.L^{-1}	Nentwig, 2007
	400 μg.L^{-1}		LC_{50}		
***Oncorhynchus* mykiss (rainbow trout)**	1545 mg.L^{-1}	24 hours	EC_{50}	• Induction of hepatocyte cytotoxicity	Laville *et al.*, 2004
	0 -140 μg.L^{-1}	4 hours		• Induction of ROS production in fish hepatocytes PLHC-1	
	2 mg.L^{-1}	48 hours	LC_{50}		Ferrari *et al.*, 2004
***Pimephales promelas* (fathead minnow)**	705 mg.L^{-1}	48 hours	LC_{50}		Brooks *et al.*, 2003
	212 μg/L R- FLX	48 hours	LC_{50}		Stanley *et al.*, 2007
	16.1 (±20.2) μg.L^{-1} R- FLX	15 minutes after 7 days exposure	EC_{10}	• Decrease of feeding rate	
	132.9 (±21.2) μg.L^{-1} R-FLX	7 days		• Decrease of growth rate	
	125 ng.L^{-1}	12d post-hatched	behavior	• Slower predator avoidance behaviors in larvae	Painter *et al.*, 2009
***Oryzias latipes* (Japanese medaka)**	0, 0.1, 0.5, 1.0 and 5.0 μg.L^{-1}	4 weeks		• Abnormalities in embryo development (edema, curved spine, no pectoral fins, reduced eyes) • Increase of female circulating estradiol levels 0.1 and 0.5 μg.L^{-1} FLX	Brooks *et al.*, 2003; Foran *et al.*, 2004
	0.2 (pH 9), 1.3 (pH 8), 5.5 (pH 7) μg.L^{-1}	96 hours	LC_{50}		Nakamura *et al.*, 2008
***Danio rerio* (zebrafish)**	0.32, 3.2, 32 μg.L^{-1}	7 days	reproduction	• Ovarian levels of 17 β-estradiol (E2) decrease • Average eggs spawning reduction • Reduction of ovarian aromatase, follicle stimulating hormone receptor (FSHr), and luteinizing hormone receptor (LHr) gene expression	Lister *et al.*, 2009

Table 4.2: (Continuation).

***Thalassoma bifasciatum* (bluehead wrasse)**	injection 6µg.g^{-1}.day^{-1}	2 weeks	behavior	• Decrease of territorial aggression	Perreault *et al.*, 2003; Semsar *et al.*, 2004
***Ictalurus punctatus* (channel catfish); Pomoxis nigromaculatus (black crappie); Lepomis macrochirus (bluegill)**	collected from 100 m downstream from the effluent discharge (Pecan Creek, USA)		bioaccumulation	• in brain (1.58 ± 0.74 ng.g^{-1}); liver (1.34 ± 0.65 ng.g^{-1}) and muscle (0.11 ± 0.0365 ng.g^{-1})	Brooks *et al.*, 2005

To our knowledge, this is the first study focused on the potential antioxidant alteration status of an environmental realistic concentration of FLX (75 $ng.L^{-1}$) exposure in mussels *M. galloprovincialis* through the assessment of antioxidant enzyme activities: SOD, CAT; Phase II GST activity, and LPO in mussels' gills and digestive gland. In parallel, SSRI FLX potential to cause neurotoxic effects response was tested by assessing the activity of an essential neurotransmission modulator, enzyme acetylcholinesterase (AChE) in mussel gills. AChE activity has been reported to be inhibited in the presence of several organic contaminants (such as pesticides, detergents and pharmaceuticals) (Almeida *et al.*, 2010; Solé *et al.*, 2010). Finally, alkali-labile phosphate (ALP) method was applied on sex-differentiated mussel gonads to assess FLX as an endocrine disruption inducer, since ALP levels are positively correlated with those from vitellogenin-like proteins which are naturally synthesized in females and inactive in males (Blaise *et al.*, 1999; Gagné *et al.*, 2002; Matozzo *et al.*, 2008).

4.2 Materials and Methods

4.2.1 Chemicals

R-(-) fluoxetine hydrochloride (F1678, > 98%, CAS: 114247-09-5); 1.1.3.3. tetramethoxypropane (MDA) (108383, CAS: 102-52-3); 1-methyl-2-phenylindole (99%, CAS: 3558-24-5); 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (D8130, ≥ 98% TLC, CAS: 69-78-3); acetyl thiocholine iodide (ATC) (A5751, ≥ 98% TLC, CAS: 1866-15-5); bovine albumin serum (BSA) (A9418 > 98%, CAS: 9048-46-8); butylated hydroxytoluene (BHT) (B1378, ≥ 99.0% GC, CAS: 128-37-0); cytochrome *c* from equine heart (C7752, > 95%, CAS: 9007-43-6); diethylenetriaminepentaacetic acid dianhydride (DTPA) (D6148, CAS: 23911-26-4); ethylenediaminetetraacetic acid (EDTA) (ED, ≥ 99%, CAS: 60-00-4); Fiske and Subbarow Reducer (F5428); glutathione reductase (G3664, CAS: 9001-48-3); HEPES (H3375, >99.5%, CAS: 7365-43-9); hydrogen peroxide solution (H1009, 30% w/w, CAS: 7722-84-1); hypoxanthine (H9377, >99%, CAS: 68-94-0); L-glutathione oxidized (GSSG) (G4501, > 98%, CAS: 27025-41-8); L-glutathione reduced (GSH) (G4251, > 98%, CAS: 70-18-8); methanesulfonic acid (>99.5%, CAS: 75-75-2); triton x-100 (X6878, CAS: 9002-93-1) xanthine oxidase from bovine milk (X1875, CAS: 9002-17-9); β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADPH) (N8129, ≥ 97%, CAS: 606-68-8) were purchased from Sigma-Aldrich (Germany). Protein-assay dye reagent concentrate

(phosphoric acid CAS: 7664-38-2 + methanol CAS: 67-56-1) was obtained from Bio-Rad Laboratories, Inc. (USA). 1,4-dithiothreitol (DTT) (≥ 99%, CAS: 3483-12-3); acetonitrile (99.8%, CAS 75-05-8); methanol (99.9%, CAS 67-56-1); natriumazide (sodium azide) (106688, ≥ 99%, CAS: 26628-22-8); potassium chloride (KCl) (104936, 99.5%, CAS: 7447-40-7); trichloroacetic acid (TCA) (100807, CAS: 76-03-9); TRIS-(hydroxymethyl)-aminomethane (≥ 99%, CAS: 77-86-1) were acquired from Merck (Germany). 1-chloro-2,4,-dinitrobenzene (CDNB) (24440, ≥ 98.0% GC, CAS: 97-00-7), molybdate reagent solution (puriss p.a.) and potassium dihydrogen phosphate (60218, 99.5%, CAS: 7778-77-0) acquired from Fluka. D(+) saccharose extra pure (16104, 99%, CAS: 57-50-1); sodium chloride (NaCl) (puriss p.a., CAS: 7647-14-5); sodium hydroxide (NaOH) (purified, CAS: 1310-73-2) obtained from Riedel-de-Haën (Germany).

4.2.2 Fluoxetine Exposure Assay (75 ng.L^{-1})

Mussels *M. galloprovincialis* (n = 245, average shell length size: 67 ± 2 mm, width: 37 ± 1 mm) were collected in 2010 from Ria Formosa Lagoon in Portugal. These specimens were transported alive to the laboratory, subjected to shell cleaning and finally placed in separate aquaria (n = 35, 1 mussel.L^{-1}) concerning control and exposure treatment to 75 ng.L^{-1} of FLX. Previously to the 75 ng.L^{-1} of FLX exposure all mussels were kept for 7 days acclimatizing in aerated natural seawater. The aquaria were kept at constant temperature (18.6 ºC ± 1), salinity (33 ± 0.4), pH (8.1 ± 0.2) and oxygen saturation (> 98% ± 2). Mussels were not fed until the end of the experiment. Water was changed and FLX concentration was re-established at every 48 hours.

At each set up time (0, 3, 7, and 15 days), mussels (n = 20) were removed from control and exposure aquaria, individual shell biometric data was measured (length, width) and dissection performed, separating gills, digestive gland and gonads. Each tissue sample was immediately frozen in liquid nitrogen and stored individually at -80ºC prior to analysis. For condition index (CI) estimation, 15 mussels of each aquarium, where individual weighted regarding the ratio:

$$CI = \frac{\text{whole soft tissue (wet weight)}}{\text{whole body tissue w/shell (wet weight)}} \text{ x } 100$$

4.2.3 Antioxidant Enzymes Analysis

Antioxidant enzyme analysis was performed using previously dissected gills (n = 5) and digestive glands (n = 5) separately. Each tissue sample was homogenized on ice individually with 20 mM TRIS buffer (containing 1 mM of EDTA + 0.5 M of saccharose + 0.15 M of KCl + 1 mM of DTT) at pH 7.6. Homogenates were centrifuged for 15 minutes at 500 × *g* at 4ºC and resulting supernatants recentrifuged for 45 minutes at 12,000 ×*g* at 4ºC. After cytosolic fraction volume measurement, Sephadex® G-25 gel columns were applied to further purify the sample removing low molecular weight proteins. Prior to the purification a 100 µl aliquot was collected for total protein quantification using bovine serum albumin (BSA) as a standard according to Bradford's method (Bradford, 1976).

Purified aliquots of gills and digestive glands were individually analyzed in triplicate for the quantification of several antioxidant enzymes by spectrophotometric analysis applying the following methodology. In order to determine SOD activity in mussel tissues, 100 µl of each purified aliquot was evaluated measuring the cytochrome *c* absorbance reduction by 50% at 550 nm wavelength generated by xanthine oxidase/hypoxanthine system according to McCord and Fridovich (1969) method. SOD activity is expressed by arbitrary units (U) per minute of mg^{-1} of total protein. For CAT activity assessment 100µl of each purified tissue aliquot was used to measure the absorbance decrease related to hydrogen peroxide (H_2O_2) consumption at 240 nm (Greenwald, 1985) and expressed as $\mu moles.mg^{-1}$ of total protein $concentration.min^{-1}$. GST activity analysis was performed quantifying the reaction of 50 µl of each purified sample with CDNB at 340 nm, following an adaptation of Habig *et al.* (1974) method and expressed as µmol of resulting CDNB conjugate $formed.mg^{-1}$ of total $protein.min^{-1}$.

4.2.4 LPO Analysis

Dissected gills (n = 10) and digestive glands (n = 10) were homogenized on ice individually with 20 mM TRIS-HCl buffer and butylated hydroxytoluene (BHT) in a 100:1 µl ratio at pH 8.6. In order to precipitate the cytosolic fraction, the homogenates were centrifuged at 30,000 × *g* for 45 minutes at 4ºC. An aliquot was set aside for total protein quantification according to Bradford's method (Bradford, 1976). Resulting cytosolic fraction was used for the measurement of LPO levels by the quantification of

by-products malondialdehyde (MDA) and (2E)-4-hydroxy-2-nonenal (HNE) formation absorbance at 586 nm following an adaptation of Erdelmeier *et al.* (1998) method. LPO levels are expressed as μmol MDA.g^{-1} total protein.

4.2.5 AChE Analysis

Dissected gills (n = 5) were homogenized in 100 mM Tris-HCl buffer and 100:1 μl of Triton at pH 8.0 on ice. The homogenates were centrifuged at 12,000 × *g* for 30 minutes at 4ºC. Resulting supernants were separated in aliquots, one for total protein determination (Bradford, 1976) and the other for AChE activity analysis according to an adaptation of Ellman *et al.* (1961) method. AChE activity was measured through the increase of yellow color resulting from the production of 5-mercapto-2-nitrobenzoate at 405 nm (ε = 13.6 $mM^{-1}.cm^{-1}$) by the reaction of substrate thiocholine with DTNB. AChE activity is expressed nmol.mg^{-1} total protein.min^{-1}.

4.2.6 ALP Analysis

Sex-differentiated gonads (n = 10) were homogenized in 25 mM Hepes–NaOH buffer (containing 125 mM NaCl + 1 mM DTT + 1 mM EDTA) at pH 7.4 in ice following an adaptation of Blaise *et al.* (1999) method. Homogenates were centrifuged at 12,000 × *g* at 2ºC for 30 minutes, resulting pellets were discarded. An aliquot of each supernant was reserved to determine total protein content (Bradford, 1976). The remaining cytosolic fractions were adjusted with 35% acetone and centrifuged at 10,000 × *g* for 5 minutes. Resulting pellets were dissolved with 1 M NaOH and placed for 30 minutes in a 60ºC heating bath. Phosphomolybdenum method (Stanton, 1968) was used to calculate inorganic phosphate (KH_2PO_4) concentration at 660 nm wavelength. ALP concentrations are expressed as μg [PO_4].mg^{-1} total protein.

4.2.7 Statistical Analysis

All biomarkers results are presented as mean ± standard deviation corresponding to each set of time. Two-way ANOVA was performed using SIGMAPLOT® to test differences between non- and mussels treated with FLX on each tissue at each set of time. Ad-hoc Holm-Sidak was used on single biomarker difference discrimination over time, within and when applicable between tissues. Pearson correlation was used to verify the

dependency between biomarkers. PCA were performed with XLSTAT® 2011 to assess each biomarker responsibility on the variability at each set of time in non- and FLX exposed 1) gills, 2) digestive glands and 3) gills vs. digestive glands. AChE activity was only considered in gills' PCA, and ALP was not considered as it was analyzed in gonads. Statistical significance was defined at $p < 0.05$ level.

4.3 Results

4.3.1 CI

A significant decrease was observed in control mussels from the beginning (26.0 ± 2.6%) to the 3rd day (22.2 ± 3.3%), remaining unchanged until the end of the experiment (min. 19.5 ± 4.4%). Additionally, FLX exposed mussels' condition index was not affected throughout the exposure (day 3: 21.9 ± 4.0; day 7: 22.0 ± 3.3; day 15: 20.0 ± 3.8%) being not significantly different from respective controls.

4.3.2 Antioxidant Enzymes

SOD activity is significantly higher in gills than in digestive glands for both non- and FLX exposed mussels throughout the experiment. Exposed gills showed a SOD activity inhibition trend over time only significant after two weeks (Figure 4.1A) ($p < 0.05$), whereas in the exposed mussels' digestive glands no significant differences with controls exist (Figure 4.1B) ($p > 0.05$).

Contrarily to SOD, CAT activity was higher in mussels' digestive glands than in gills. Even though, no significant differences between control and FLX exposed gills were established, it was noticeable a slight CAT activity increase in exposed gills, which was related to the SOD activity inhibition over time in this tissue ($r = -0.921$, $p < 0.05$) (Figures 4.1 C and D). This was further related with an inverse relationship between these antioxidant enzymes. In digestive gland, controls mussels showed a slight, but significant CAT decrease over the experiment duration, while exposed mussels showed a transient pattern, in which CAT activity fluctuates to significantly higher activity levels than controls after 3 and 15 days of FLX exposure ($p < 0.05$).

Regarding Phase II enzyme GST (Figures 4.1E and F), both tissues exhibit similar activities, although with irregular patterns. In controls, GST activities in both tissues decreased; after the first week in gills and immediately after the 3rd day in digestive gland. In exposed mussels, GST activity remained unaltered in gills (Figure 4.1E), whereas in digestive gland (Figure 4.1F) it fluctuates decreasing to control levels after the first week of exposure ($p < 0.05$) and varying to significantly higher activities than controls after the 3rd and 15th day of exposure. Furthermore, GST and CAT activities are directly related in this tissue ($r = 0.791$, $p < 0.05$).

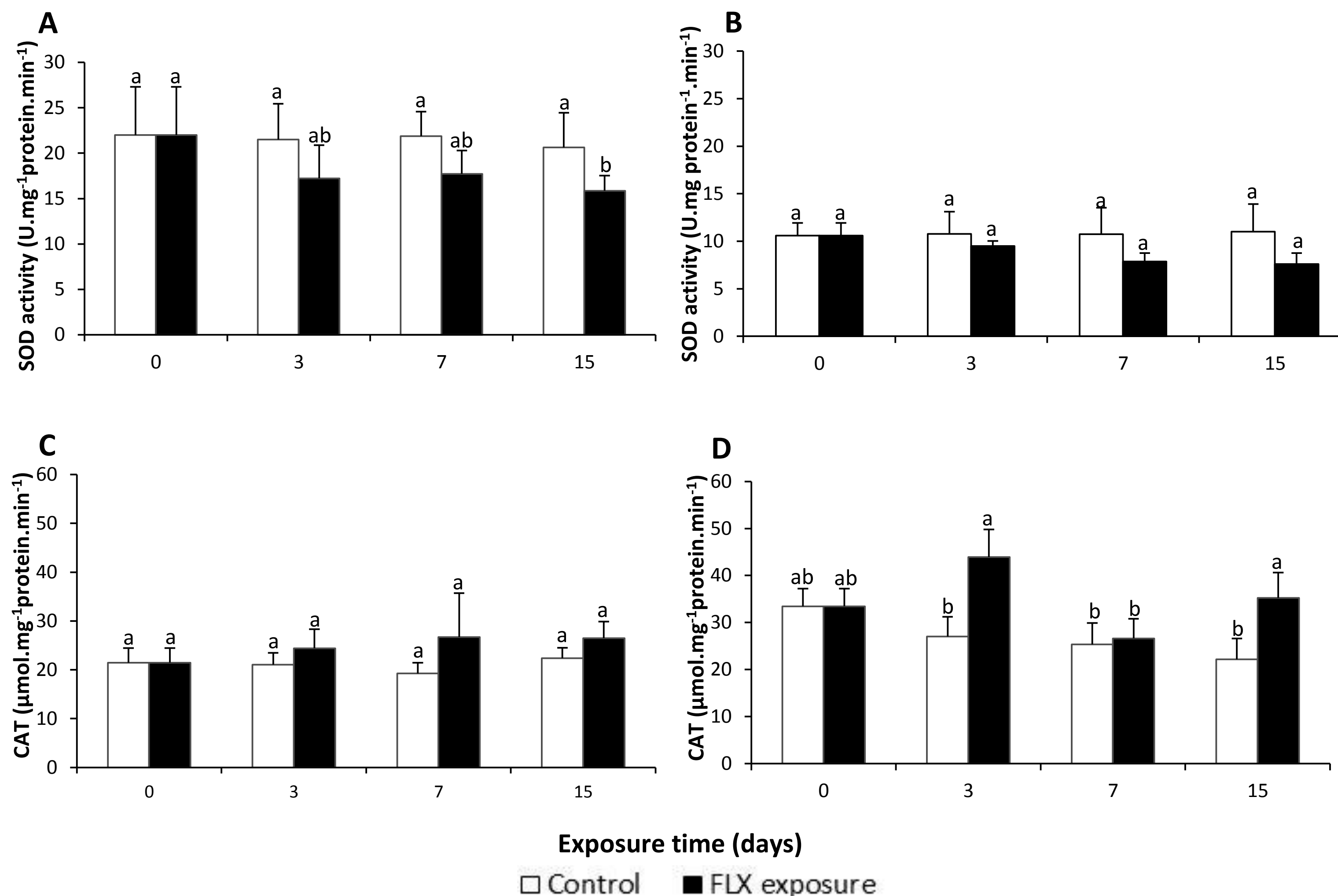

Figure 4.1: Antioxidant enzymes activity (mean ± standard deviation) in control and FLX exposed *M. galloprovincialis* tissues. SOD activity in gills (A) and in digestive gland (B); CAT activity in gills (C) and in digestive gland (D). Different letters express significant differences ($p < 0.05$). Note: Figure continues in the next page.

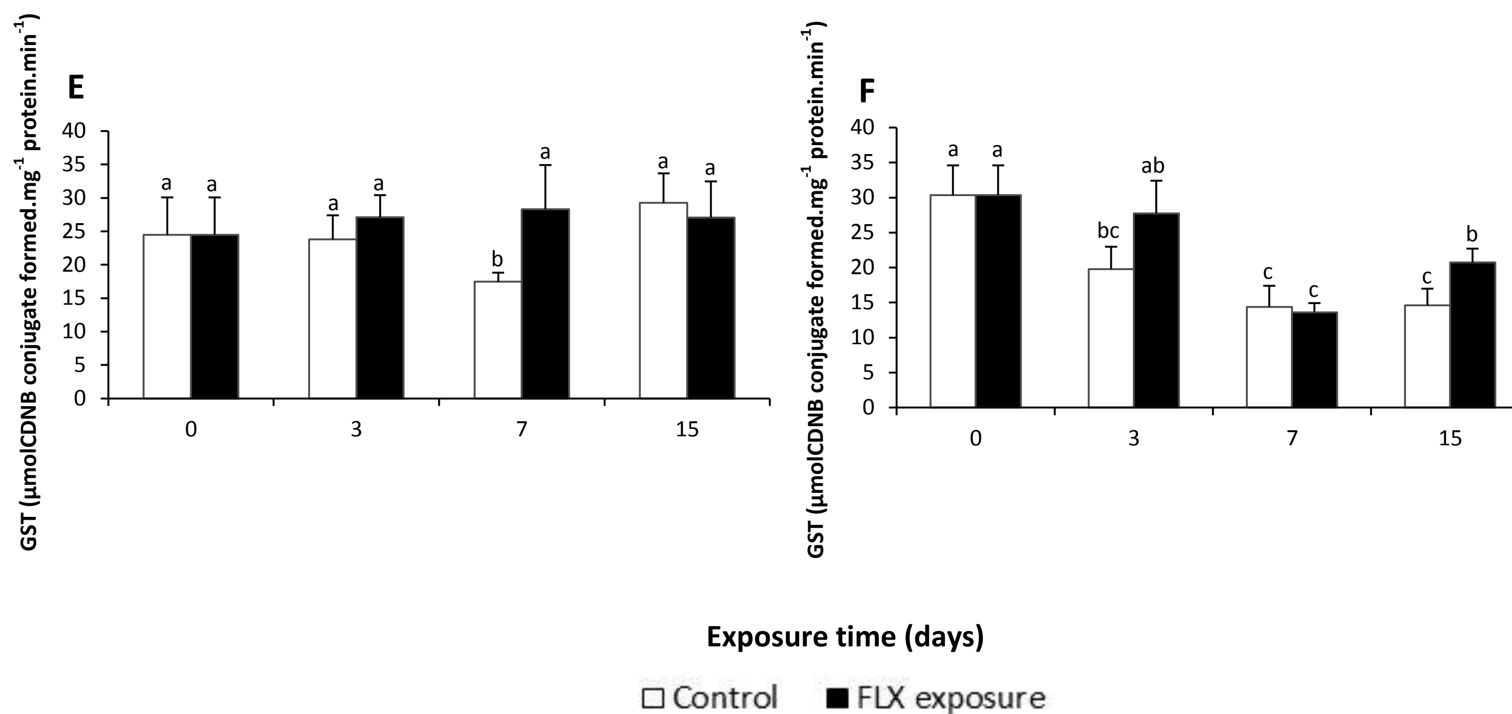

Figure 4.1 (Continuation).: Antioxidant enzyme activity (mean ± standard deviation) in control and FLX exposed *M. galloprovincialis* tissues. GST activity in gills (E) and digestive gland (F). Different letters express significant differences ($p < 0.05$).

4.3.3 LPO

LPO levels (Figures 4.2A and B) were higher in gills than in digestive glands in both mussels groups. In gills (Figure 4.2A), while controls showed no differences over time ($p > 0.05$), exposed mussels exhibit a significantly higher LPO levels than controls after one week ($p < 0.05$), recovering to control levels by the end of the experiment ($p > 0.05$). In the digestive gland (Figure 4.2B), controls remained unaltered after a significantly decrease at the 3rd day, whilst exposed mussels LPO levels increased but only significantly after two weeks of exposure (4-fold higher than controls) ($p < 0.05$).

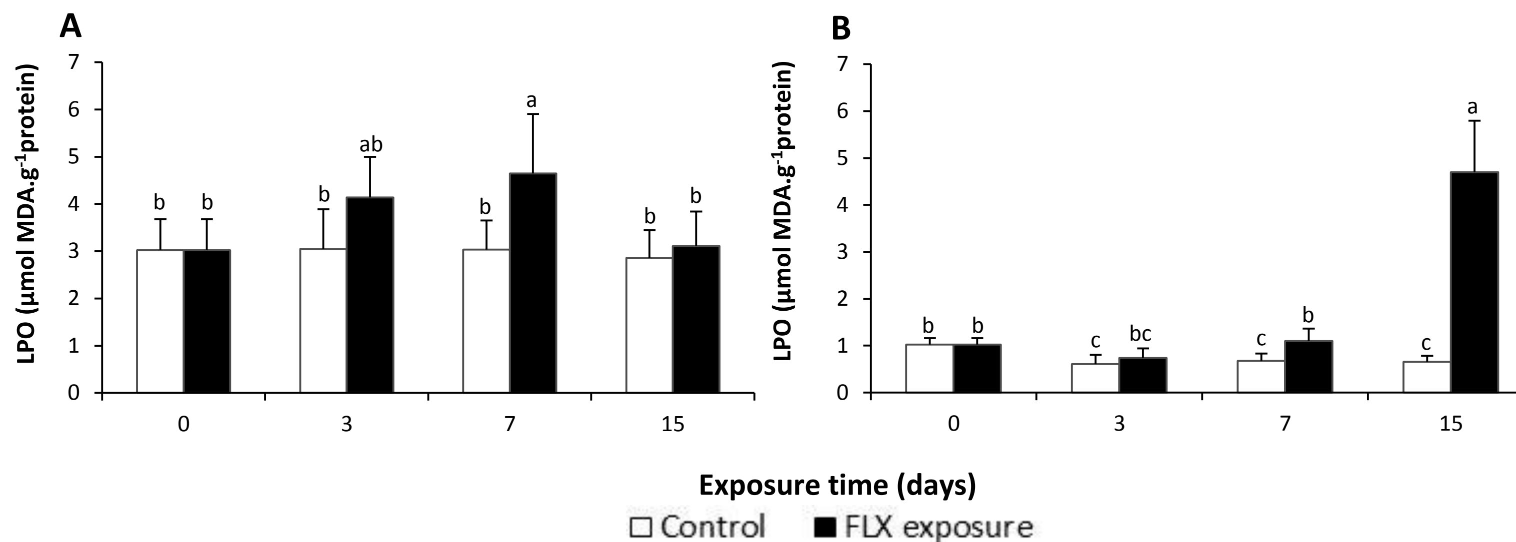

Figure 4.2: LPO (mean ± standard deviation) in control and FLX exposed *M. galloprovincialis* tissues, in gills (A) and digestive gland (B). Different letters express significant differences ($p < 0.05$).

4.3.4 AChE

AChE (Figure 4.3) activity remained consistent in control individuals throughout the experiment ($p > 0.05$). Conversely AChE activity was significantly incremented in mussels' gills after the 3rd day of FLX exposure comparing to controls, followed by a progressive inhibition, reaching a significantly lower activity than controls by the end of the experiment ($p < 0.05$).

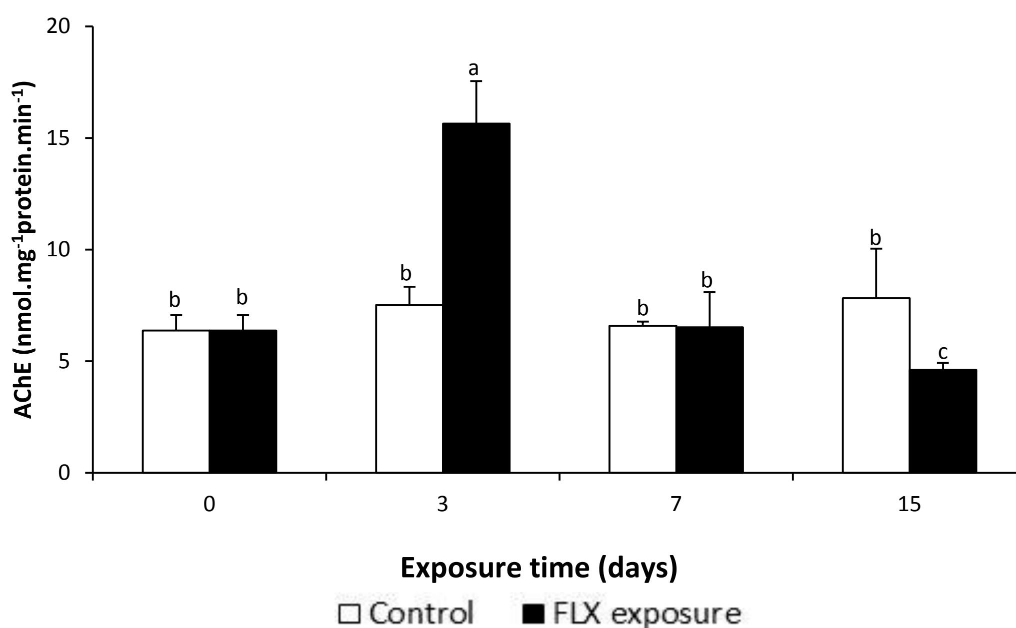

Figure 4.3: AChE activity (mean ± standard deviation) in non- and exposed mussels' *M. galloprovincialis* gills to 75 ng.L^{-1} of FLX along 15 days. Different letters express significant differences ($p < 0.05$).

4.3.5 ALP

ALP levels (Figure 4.4) decreased significantly in control females after the first week ($p < 0.05$), while in males' remained unchanged throughout the experiment duration ($p > 0.05$). Nevertheless in exposed gonads, after 3 days of exposure, ALP levels decreased significantly in both females and males though more markedly in males comparing to controls. After this, ALP levels were significantly incremented after one week of exposure in both genders, decreasing again to levels significantly lower than controls by the end of the experiment ($p < 0.05$).

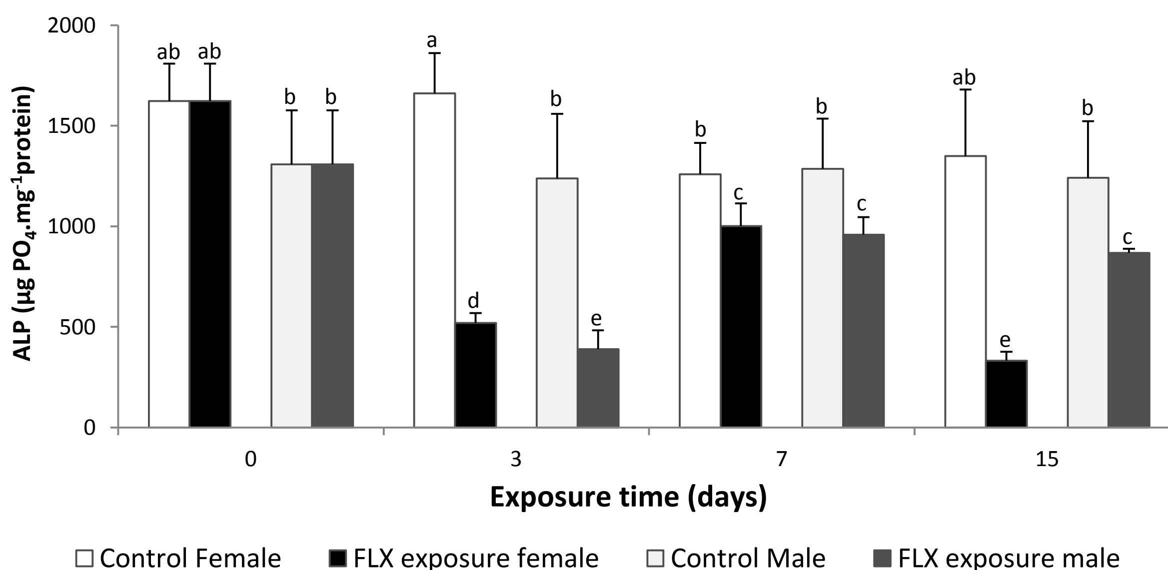

Figure 4.4: ALP level (mean ± standard deviation) in non- and exposed mussel's gonads *M. galloprovincialis* to 75 ng.L^{-1} of FLX throughout 15 days. Different letters express significant differences ($p < 0.05$).

4.3.6 PCA in Gills and Digestive Gland

PCA was applied to all parameters measured in the gills (Figure 4.5A) revealing 72 % of total variance (TVar). PC1 represents 52% of variance, highlighting the separation between non- and FLX exposed gills by the opposition in the factorial weight distribution of SOD activity with the remaining enzymes activities and LPO levels. The second component (explaining only 19% of the variance) shows the further influence of CI in the separation of day 0 (control) from the remaining controls, and day 15 FLX exposed gills from the other exposure-day groups in opposition to LPO levels and AChE activities. Furthermore, PCA reveals that SOD and CAT activities exhibit opposing factorial distribution, corroborating with the above mentioned inverse relationship.

Digestive gland PCA (Figure 4.5B) represents almost 84% of TVar, in which both components had similar influence on the overall groups' distribution (PC1, 46% and PC2, 38%). AChE activity was not considered since it was performed only in gills. PC1 shows the cluster of controls (exception for day 0) particularly associated to the opposition of SOD activities with the remaining variables; and the separation of day 7 FLX exposed digestive glands from the other exposure days. As observed in gills PC2, CI is the highest factor explaining the variability of day 0 control group from remaining groups and in this case, day 15 FLX exposed digestive glands were further influenced by the LPO levels. The digestive gland PCA also corroborates with the direct relationship between CAT and GST activities in this tissue.

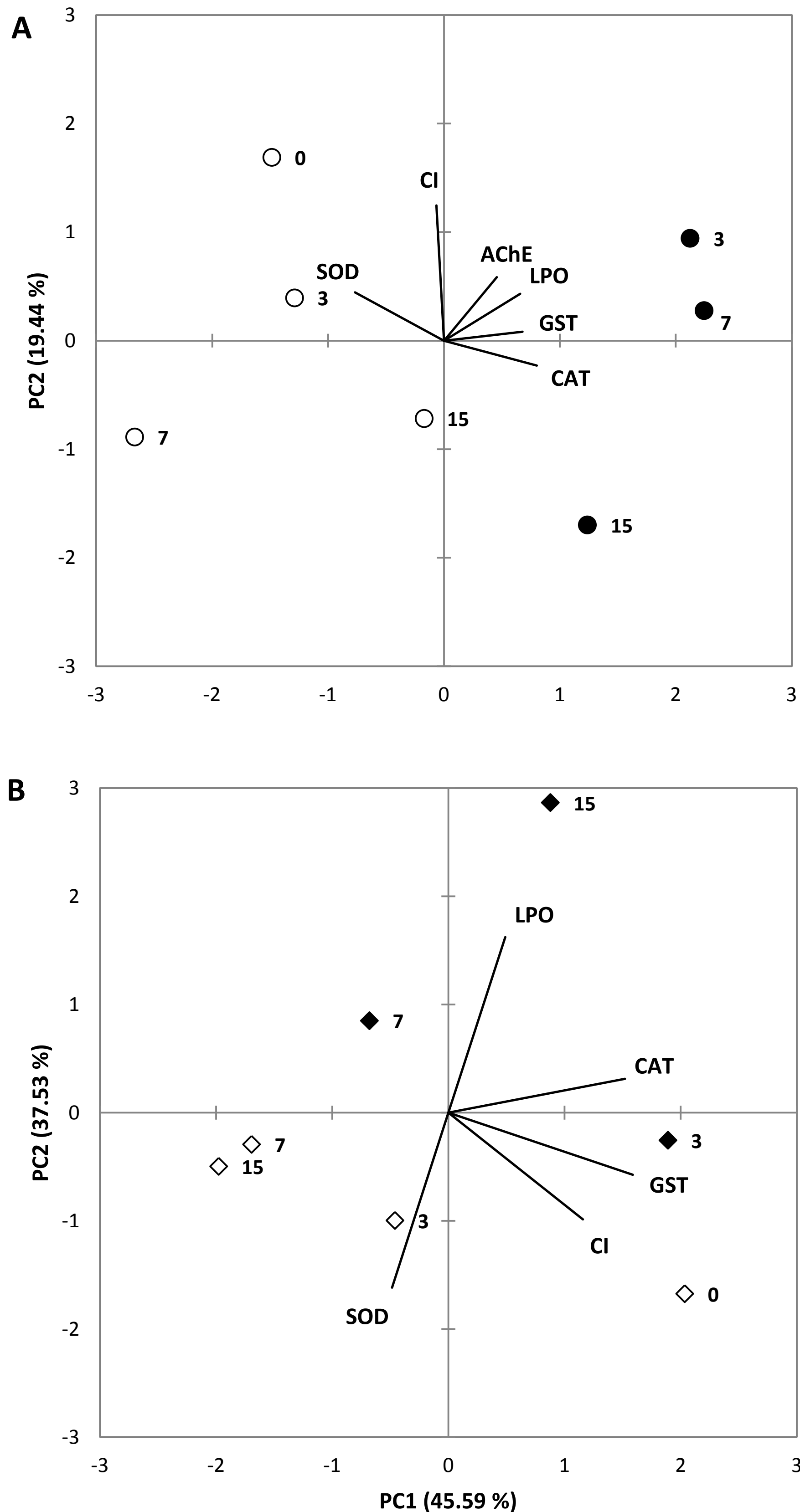

Figure 4.5: PCA equivalent to the integration (A) of SOD, CAT and GST, LPO, AChE and CI on 77% of total variance in gills; - (B) of SOD, CAT and GST, LPO and CI on 83% of total variance in digestive gland of mussel *M. galloprovincialis* between treatments (●, ♦ – control, ○, ◊ – FLX exposed mussels) over exposure time (days: 0, 3, 7 and 15).

PCA (Figure 4.6) is related with tissue-specific integration of oxidative stress biomarkers (SOD, CAT, GST and LPO), explaining approximately 85% of TVar (PC1, 51% and PC2, 34%). The first component clearly separates non- and FLX exposed gills from digestive glands treatment groups, particularly regarding gills to LPO levels and GST and SOD activities, while digestive glands are more related to the activity of CAT. The second component highlights the separation of both exposed gills and digestive glands from controls groups, with the exception of day 0 control and day 7 exposed digestive glands and day 15 gills control regarding to levels of CAT, LPO and GST in opposition to SOD. In overall PCA shows the aggregation of: 1) gills controls relating to higher SOD activities; 2) FLX exposed gills with the increment of LPO levels; 3) FLX exposed digestive glands with higher CAT activities; and finally 4) digestive glands controls in opposition to all integrated biomarkers.

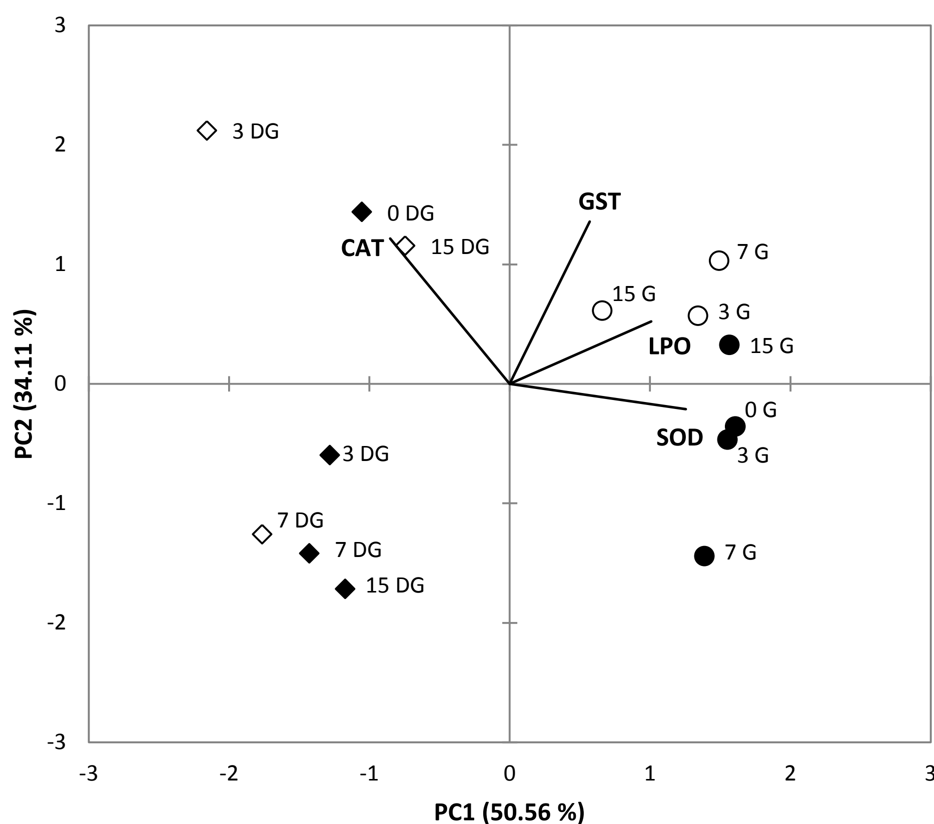

Figure 4.6: PCA related to the integration of SOD, CAT and GST, LPO on 85% of total variance in mussels´ *M. galloprovincialis* tissues between treatments (● – gills controls, ○ – FLX exposed gills, ♦ – digestive gland controls, ◊ – FLX exposed digestive gland) over exposure time (days: 0, 3, 7 and 15).

4.4 Discussion

4.4.1 Oxidative Stress

To our knowledge this is the first study concerning the effect of FLX as a potential oxidative stress inducer applying antioxidant enzymes response activities in mussels. The results reveal a transient antioxidant status alteration in both mussels' tissues. As the first organ in direct contact with the contaminant, the significant SOD activity down regulation was inversely related only in the gills, the concomitant inhibition tendency of SOD activity in digestive glands resulted in a significant enhancement of CAT after 3 days and two weeks of FLX exposure (Figures 4.1). The exposure of rainbow trout (*Oncorhynchus mykiss*) hepatocytes to FLX has shown to induce an enhancement of ROS production (Laville *et al.*, 2004). Considering SOD primarily role in the dismutation of $OH^{\cdot-}$ into H_2O_2, its inhibition in presence of FLX is associated to a higher presence of ROS particularly in the gills and since digestive gland has a higher participation on general redox-cycling and biotransformation processes (Livingstone *et al.*, 1992) CAT's catalytic action is later triggered in this tissue (Regoli and Principato, 1995). An inhibition of SOD activity was also found by Djordjevic *et al.* (2011) in mice exposed to 5 $mg.kg^{-1}$ FLX body mass, although no enhancement of CAT activities was observed. Additionally, higher CAT activities were reported in digestive glands than in gills in the same mussel species exposed to carbamazepine (Martín-Díaz *et al.*, 2009). Overall PCA clustering (Figure 4.6) provides a further validation for the discrepancy between the activity levels of SOD in gills and CAT in digestive gland.

Phase II GST promotes reduced glutathione (GSH) conjugation with parental electrophilic compounds enabling its transformation to more extractable hydrophilic metabolites (Halling-Sørensen *et al.*, 1998). Although, GST activity was generally transitory between treatments in both tissues (Figures 4.1E and F) giving evidence to the actuation of other affecting variables rather than exposure itself, this enzyme was more responsive in FLX-exposed digestive gland showing simultaneously higher activity than controls (except after one week) and was directly related with CAT activity. A positive relationship between the enhancement of GST and CAT was also observed in *M. galloprovincialis* digestive glands exposed to 250 $ng.L^{-1}$ ibuprofen for the same time (Gonzalez-Rey and Bebianno, 2012 – Chapter 3B) alike for bezafibrate (Canesi *et al.*, 2007), carbamazepine (Martín-Díaz *et al.*, 2009) and propanolol exposure

(Franzellitti *et al.*, 2011). Finally, GST enhancement confirms, as stated by Canesi *et al.* (2007) after 38.2 ng.g^{-1} bezafibrate injection in same species mussel, that FLX is partially metabolized in mussels' digestive glands.

The alteration of antioxidant enzymes was unable to prevent and counteract FLX-exposure as there was a clear tissue-specific response of membrane damage in which gills showed higher vulnerably at the beginning of FLX-exposure until the end of the first week while LPO enhancement in exposed digestive glands (7-fold higher) continued until the end of the experiment (Figures 4.2A and B). Even though, exposed gills seemed to recover to control levels after 2 weeks, PCA (Figure 4.6) suggests that FLX has a higher impact in this tissue than in digestive gland over time. Higher levels of LPO were also observed after 250 ng.L^{-1} ibuprofen exposure in the same mussels' tissues particularly in gills (max: 19 µmol MDA mg^{-1} protein) than the ones induced by FLX (Chapter 3A-B - Gonzalez-Rey and Bebianno, 2011; 2012).

4.4.2 Neurotoxic Effect

AChE is responsible for the hydrolysis of neurotransmitter acetylcholine (ACh) to choline and acetic acid and has an important role in cholinergic nervous function (Tsuchiya *et al.*, 2004), as the neurotransmitter 5-HT has in serotonergic neurotransmission. These neurotransmitters are critical in the control of many physiological processes like e.g. cardiac regulation in bivalves, though their roles may be inverted regarding excitation and inhibition in different species (Kuwasawa and Hill, 1997). Since SSRI FLX induces an increase in extracellular 5-HT by the serotonin transport protein inhibition at nerve synapses (Lister *et al.*, 2009) is not illicit to think that AChE may also be affected by FLX presence, further considering the evidences of 5-HT increase in mussel *E. complanata* after injection with FLX (Gagné and Blaise, 2003).

Even though AChE activity has been reported to be inhibited in mussels by APIs namely by paracetamol (23 and 403 µg.L^{-1}) in *M. galloprovincialis* gills (Solé *et al.*, 2010) and by diazepam (4, 20 and 100 nmol per mussel) in *E. complanata* visceral mass (Gagné *et al.*, 2011) in FLX-exposed *M. galloprovincialis* gills the significant down regulation of AChE activity was only observed at the end of the 15th day, being firstly preceded by a clear up regulation of AChE after 3 days of FLX exposure. Few other studies have shown the induction of AChE namely, in mussels placed in the final

aeration lagoon (Gagné *et al.*, 2010) and in crabs in an impacted stream (Nieto *et al.*, 2010) with no noticeable explanation for this fact rather than antagonistic effects between contaminants. The enhancement of AChE activity implies the depletion of ACh, we hypothesize several possible explanations for the AChE activity temporary enhancement at the beginning of the experiment: 1) the enhancement of neurotransmitter 5-HT concentration by FLX action at nerve endings may have been competing with ACh and therefore causing its depletion, even though the receptor activation mechanism of 5-HT in mussels is still unknown as stated by Gagné and Blaise (2003); 2) AChE increase is associated to cell apoptosis (Zhang *et al.*, 2002), FLX presence may promote gills' cell apoptosis at the beginning of the experiment followed by the mussels' recovery to controls' AChE activity; 3) A relationship between estradiol levels and AChE activity regulation in mice brain cortex cells was reported noting that with high levels of 17β –estradiol (E2) AChE activity was suppressed whereas without E2, it increased (Tsuchiya *et al.*, 2004). The alteration of endogenous levels of E2 may explain these AChE activity alterations in FLX exposed mussels at the beginning of the exposure, even though AChE activity was not measured in sex-separated gills the results indicate by the concomitant ALP down regulation over the 3rd day of exposure that FLX has the ability to interfere with estrogen receptors.

4.4.3 Endocrine Disruption

ALP levels are released by vitellogenins (Vtg) after alkali hydrolysis (Porte *et al.*, 2006), which precede egg-yolk protein vitellin (Vn) in oviparous species (Matozzo *et al.*, 2008). In bivalves, vitellogenesis is induced by estradiol (E2) and a neuropeptide (Matozzo *et al.*, 2008). ALP levels enhancement is a sign of endocrine disruption (ED) in males (Blaise *et al.*, 1999; Gagné *et al.*, 2002; Matozzo *et al.*, 2008). In FLX exposure, ALP levels were generally high (max. observed in females 1600 µg [PO_4].mg^{-1} total protein) and similar to the ones in *M. edulis* in anthropogenic influenced sites (Gagné *et al.*, 2008). However FLX exposure did not induce an enhancement of males ALP levels, but rather a down regulation in both sex-differentiated gonads particularly in females over the 3rd day. A similar experimental design with the same species exposed to 250 ng.L^{-1} of ibuprofen showed both: lower basal ALP values (max. in exposed males: 117 µg [PO_4].mg^{-1} total protein) and significant induction in exposed mussels (Chapter 3B - Gonzalez-Rey and Bebianno, 2012).

ALP down regulation was also observed in females exposed to North Sea crude oil (NSO) which in turn was accompanied by the decreased of gonads development suggesting ALP levels influence on gonad development in adult blue mussels *M. edulis* through an antiestrogenic effect of polycyclic aromatic hydrocarbon (PAHs) and oils (Ortiz-Zarragoitia and Cajaraville, 2006). As also stated by these authors it is important to consider 5-HT function in bivalves on gonad development and reproduction, for this reason a histological study of FLX-exposed gonads would greatly complement the data on mussels' reproduction fitness alteration.

Furthermore, the ALP down regulation in both FLX-exposed female and male gonads may be associated to E2 inverse relationship with 5-HT levels as reported in freshwater mussels *E. complanata* and gonad 5-HT concentration increase during spawning (Gagné and Blaise, 2003) since by opposition Vtg synthesis induction is related to gonad 5-HT levels decrease (Matozzo *et al.*, 2008). Also, recently Lazzara *et al.* (2012) reported the increase of 1.5-fold of E2 after 6 days of exposure to 200 ng.L^{-1} of FLX in mussel *D. polymorpha*. Nevertheless, along with the decrease of oocytes and spermatozoan, esterified E2 levels decreased approximately 3 to 8-fold in this species between spawning and after-spawning phases after 20 and 200 ng.L^{-1} of FLX exposure thus confirming this SSRI to be an endocrine disruptor.

4.5 Conclusions

FLX exposure to mussels for two weeks induced a transient antioxidant enzyme activities alteration particularly in gills. However these alterations were not as severe as overall ALP sex-differentiated down regulation in gonadal tissues, highlighting a higher endocrine disruption effect of FLX rather than an oxidative stress inducer. Furthermore, resulting from the influence of FLX as a SSRI, on 5-HT levels increase as mentioned before, AChE activity was clearly altered throughout the experiment and possibly results on cholinergic neurotransmission functions breakdown. The measurement of FLX concentration in tissues, as well as, serotonin and estradiol level alteration in both non- and exposed mussels' tissues should clearly complement these findings. Finally, FLX presence, even at a relevant environmental concentration, clearly has potential to induce ecotoxicological effects in mussel *M. galloprovincialis* particularly affecting its reproduction fitness, although the mechanisms of 5-HT receptor activation in mussels are still unknown.

4.6 References

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Chapter 5

Effects of NSAID diclofenac in mussel *M. galloprovincialis*

5 *Effects of Non-Steroidal Anti-Inflammatory drug (NSAID) Diclofenac Exposure in Mussel* Mytilus galloprovincialis

Abstract

An increasing research effort has been made focused on the assessment of active pharmaceutical ingredients (APIs) occurrence in the aquatic system. Yet a lot is still to unveil concerning its potential effects in non-target organisms due to the complexity of each specific drug mode of action, reactivity and bioconcentration potential. Non-steroidal anti-inflammatory drug (NSAID) diclofenac (DCF) is one of the most frequently detected in surface waters worldwide and has been recently included in the list of priority substances under the European Commission. In this study mussels *M. galloprovincialis* were exposed to an environmental relevant concentration of DCF (250 $ng.L^{-1}$) during two weeks. Several biomarkers responses were assessed in mussel tissues: condition index (CI); superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and Phase II glutathione-S-transferase (GST) activities and lipid peroxidation levels (LPO) associated to oxidative stress; acetylcholinesterase (AChE) activity related to neurotoxic effect and finally vitellogenin-like proteins linked to endocrine disruption. The results reveal significant induction of SOD and GR activities in gills and CAT activity and LPO levels in digestive glands. Phase II GST remained unaltered in both tissues while AChE activity up regulation was directly related to vitellogenin-like protein levels in exposed-females which point to an estrogenic activity alteration rather than a cholinergic neurotransmission functions breakdown. This study confirms that DCF at a concentration often found in surface water induces tissue-specific biomarker responses. Finally this study also reveals the importance of a multi biomarker approach on the potential deleterious assessment in a species extremely vulnerable to the continuously discharge of APIs into the aquatic systems providing crucial new information on the still unknown effects of DCF as a priority substance.

5.1 Introduction

Diclofenac (DCF) (known as Voltaren®) is amongst the most applied non-steroidal anti-inflammatory drugs (NSAIDs) in human and veterinary health practice due to its properties in the relieve of acute pain and osteo- and/or rheumatoid arthritis symptoms (Boesterli, 2003; Praveen Rao and Knaus, 2008; Ng *et al.*, 2008). Nevertheless, DCF is also related to cause chronic gastric and renal toxicity in humans and Indian vulture (*Gyps* sp.) population decline after scavenging DCF-treated livestock carcasses (Boesterli, 2003; Oaks *et al.*, 2004; Hinz and Brune, 2006; Taggart *et al.*, 2007a, b; Praveen Rao and Knaus, 2008; Saini *et al.*, 2012).

After intake, DCF is partially biotransformed to hydroxilated metabolites (e.g. 4'-hydroxy (OH) and 5-OH) through cytochrome P450 (CYP450) oxidation and is excreted via urine and faeces reaching wastewater treatment plants (WWTP) and ultimately the aquatic systems (Schwaiger *et al.*, 2004; Marco-Urrea *et al.*, 2010). Several authors report the presence of this API in superficial waters (river, ponds, lake and streams) with the maximum value of 15 $\mu g.L^{-1}$ found in Erft river, Germany (Jux *et al.*, 2002) (Table 5.1). Accordingly, DCF is considered as a class IIA - “potentially hazardous” compound with relevant adverse effects (Besse and Garric, 2008) and included in the list of priority substances by the European Union (European Comission, 2012a, b).

The ability of NSAIDs to promote the decrease of prostaglandins (PGs) biosynthesis from phospholipid arachidonic acid (AA) by the nonselective inhibition of cyclooxygenase (COX)-1 and -2 isoforms (Vane *et al.*, 1998; Fent *et al.*, 2006; Praveen Rao and Knaus, 2008) may interfere in critical physiological functions, like reproduction, water transport, osmoregulation and immune defense in non-target organisms (Ruggeri and Thoroughgood, 1985; Osada and Nomura, 1990; Vane *et al.*, 1998; Rowley *et al.*, 2005; Fent *et al.*, 2006). DCF-exposure in aquatic species and particularly in bivalves induces acute and/or chronic toxicity on survival, swimming and feeding behavior, growth, embryonic development, reproduction (see Nassef *et al.*, 2010a, b; Lee *et al.*, 2011; Quinn *et al.*, 2011; Schmidt *et al.*, 2011) and bioconcentration factor (BCF) (Hoeger *et al.*, 2008) at concentrations higher than the ones found in the environment. Nonetheless, in order to predict waterborne DCF-impact

at an ecotoxicological endpoint there are still limited information concerning medium-/long term chronic exposure effects and BCF at environmental realistic concentrations (Table 5.2).

Biomarkers enable an early assessment of potential contaminant-derived chronic effects in marine life through the swift alterations of several biochemical responses in a given sentinel organism (Van der Oost *et al.*, 2003). Bivalve species, such as mussels, are considered useful bioindicators species, combining both a wide distribution and long-life cycle to a natural ability to accumulate contaminants present in the surrounding water (Cajaraville *et al.*, 2000; Ortiz-Zarragoitia *et al.*, 2010; Porte *et al.*, 2006; Ricciardi *et al.*, 2006; Cravo *et al.*, 2009; Ericson *et al.*, 2010). Considering the above this study undertook a multi-biomarker approach related to: general physiology - condition index (CI), antioxidant defense system - antioxidant enzyme activities: superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and Phase II glutathione S-transferase (GST); damage - lipid peroxidation (LPO); neurotoxic effect - acetylcholinesterase activity (AChE) and vitellogenesis/endocrine disruption - alkali-labile phosphate level (ALP) exposing mussel *M. galloprovincialis* to an environmental relevant concentration of DCF (250 ng.L^{-1}) for two weeks.

Table 5.1: DCF concentrations (ng.L^{-1}) in the aquatic system

Country	Environment	Concentration (ng.L^{-1})	Reference
Austria	river	23	Ahrer *et al.*, 2001
		166	
Brazil	river	40	Stumpf *et al.*, 1999
Canada	harbor	194	Metcalfe *et al.*, 2003
	river	420	
		15	Hua *et al.*, 2006
China	river	11 – 150	Hao *et al.*, 2007
		1-52	Wang *et al.*, 2010
Croatia	surface water	60	Gros *et al.*, 2006
Finland	river	3-35	Lindqvist *et al.*, 2005
France	river	2	Rabiet *et al.*, 2006
	surface water	5	Vulliet and Cren-Olivé, 2011
		1-33	Togola *et al.*, 2008
Germany	groundwater well	380	Heberer *et al.*, 1997
	groundwater	590	Sacher *et al.*, 2001
	pond and river	11 – 15033	Jux *et al.*, 2002
	river	1200	Ternes, 1998
		50	Wiegel *et al.*, 2004
		16 - 36	Ahrer *et al.*, 2001
	surface water	1000	Heberer, 2002
		23 - 140	Letzel *et al.*, 2009
		6	Weigel *et al.*, 2002
		31 - 67	Weigel *et al.*, 2004
		1000	DeLorenzo *et al.*, 2008
Italy	river	489	Zuccato *et al.*, 2001
Korea	river	1-7	Kim *et al.*, 2007
Luxembourg	river	19 - 55	Pailler *et al.*, 2009
Pakistan	harbor lagoon	100	Scheurell *et al.*, 2009
	river	700 - 4400	
Slovenia	river	9 - 282	Kosjek *et al.*, 2005
		89	Antonic and Heath, 2007
Spain	river	49 - 332	Farré *et al.*, 2001
		1 - 156	Fernández *et al.*, 2010
		2200	Ginebreda *et al.*, 2010
Sweden	river	120 - 10	Bendz *et al.*, 2005
Switzerland	lake	n.d. - 10	Öllers *et al.*, 2001
	river	20 – 150	
		5-10	Tixier *et al.*, 2003
UK	estuary	8 - 195	Thomas and Hilton, 2004
	river	20 - 568	Ashton *et al.*, 2004
		91	Hilton and Thomas, 2003
USA	lake	17 - 42	Wu *et al.*, 2009
	stream	32	Spongberg *et al.*, 2008
various	river	26 - 72	Hernando *et al.*, 2006

Table 5.2: DCF effects in aquatic species

Species	Exposure concentration	Exposure time (days)	Biological end-points	Effects	Reference
Dreissena polymorpha	0.3, 1, 2 nM	4	cyto and genotoxicity	- no effects on apoptosis frequency, micronucleous test and lysosomal stability	Parolini *et al.*, 2010
			oxidative stress status	- no effects on SOD, CAT, GPx or GST	
	1 $\mu g.L^{-1}$	4	cytotoxicology	- cell viability decrease in gills and hemocytes	Parolini *et al.*, 2011
			physiology	- no alteration on CI	Quinn *et al.*, 2011
			oxidative stress and DNA damage	- GST, LPO and metallothionein (MT) increase, no DNA damage (visceral mass)	
			endocrine disruption	- no alteration on ALP levels (visceral mass)	
Mytilus edulis trossulus	1 $\mu g.L^{-1}$	14 – 19	physiology	- scope for growth decrease tendency (SFG)	Ericson *et al.*, 2010
		8 – 21		- byssus strength decrease tendency	
		8	BCF	- 0.18 ± 0.02 $\mu g.gww^{-1}$ (whole body)	
Mytilus spp.		4	oxidative stress and DNA damage	- GST, LPO increase, no alteration of MT and DNA damage (digestive gland)	Schmidt *et al.*, 2011
			endocrine disruption	- no alteration on ALP levels (gonads)	
Hyalella azteca	48.7 $\mu g.kg^{-1}$ (DCF-enriched sediment)	1/2 – 3	oxidative stress	- SOD, CAT, GPx activities increase	Oviedo-Gómez *et al.*, 2010
				- LPO level increase	
Carcinus maenas	10 – 100 $ng.L^{-1}$	7	physiology	- impairment of osmo- and iono regulatory ability	Eades and Waring, 2010
Oryzias latipes	1 $\mu g.L^{-1}$	4	gene expression	- CYP P450 1A, P53 and vitellogenin in males up regulation	Hong *et al.*, 2007
Oncorhynchus mykiss	1.6 $\mu g.L^{-1}$	15	gene expression	- inflammation and immune response down regulation	Cuklev *et al.*, 2011
			BCF	- 4.02 ± 0.75 (blood plasma); 2.54 ± 0.36 (liver)	
	1 $\mu g.L^{-1}$	28	BCF	- 2732 (liver), 971 (kidney), 763 (gills) higher than at ≥ 5 $\mu g.L^{-1}$	Schwaiger *et al.*, 2004
	(LOEC)		hystopathological alterations	- no organ (gills and kidney) lesions only at ≥ 5 $\mu g.L^{-1}$	

Table 5.2: (Continuation).

Oncorhynchus mykiss			cytopathology alterations	-	in liver, kidneys and gills, no alteration in the intestine	Schwaiger *et al.*, 2004
Salmo trutta f. fario	0.5 $\mu g.L^{-1}$ (LOEC)	7 – 21	immunohistological alterations	-	erythrocyte volume in % of total blood volume and leucocrit values decrease	Hoeger *et al.*, 2008
			histopathological alterations	-	in gills, kidneys and liver	

5.2 Materials and Methods

5.2.1 Chemicals

Diclofenac sodium salt (D68990, CAS: 15307-79-6); tetramethoxypropane (MDA) (108383, CAS: 102-52-3); 1-methyl-2-phenylindole (99%, CAS: 3558-24-5); 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (D8130, ≥ 98% TLC, CAS: 69-78-3); acetyl thiocholine iodide (ATC) (A5751, ≥ 98% TLC, CAS: 1866-15-5); bovine albumin serum (BSA) (A9418 > 98%, CAS: 9048-46-8); butylated hydroxytoluene (BHT) (B1378, ≥ 99.0% GC, CAS: 128-37-0); cytochrome *c* from equine heart (C7752, > 95%, CAS: 9007-43-6); diethylenetriaminepentaacetic acid dianhydride (DTPA) (D6148, CAS: 23911-26-4); ethylenediaminetetraacetic acid (EDTA) (ED, ≥ 99%, CAS: 60-00-4); Fiske and Subbarow Reducer (F5428); glutathione reductase (G3664, CAS: 9001-48-3); HEPES (H3375, >99.5%, CAS: 7365-43-9); hydrogen peroxide solution (H1009, 30% w/w, CAS: 7722-84-1); hypoxanthine (H9377, >99%, CAS: 68-94-0); L-glutathione oxidized (GSSG) (G4501, > 98%, CAS: 27025-41-8); L-glutathione reduced (GSH) (G4251, > 98%, CAS: 70-18-8); methanesulfonic acid (>99.5%, CAS: 75-75-2); triton x-100 (X6878, CAS: 9002-93-1) xanthine oxidase from bovine milk (X1875, CAS: 9002-17-9); β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADPH) (N8129, ≥ 97%, CAS: 606-68-8) were purchased from Sigma-Aldrich (Germany). Protein-assay dye reagent concentrate (phosphoric acid CAS: 7664-38-2 + methanol CAS: 67-56-1) was obtained from Bio-Rad Laboratories, Inc. (USA). 1,4-dithiothreitol (DTT) (≥ 99%, CAS: 3483-12-3); acetonitrile (99.8%, CAS 75-05-8); methanol (99.9%, CAS 67-56-1); natriumazide (sodium azide) (106688, ≥ 99%, CAS: 26628-22-8); potassium chloride (KCl) (104936, 99.5%, CAS: 7447-40-7); trichloroacetic acid (TCA) (100807, CAS: 76-03-9); TRIS-(hydroxymethyl)-aminomethane (≥ 99%, CAS: 77-86-1) were acquired from Merck (Germany). 1-chloro-2,4,-dinitrobenzene (CDNB) (24440, ≥ 98.0% GC, CAS: 97-00-7), molybdate reagent solution (puriss p.a.) and potassium dihydrogen phosphate (60218, 99.5%, CAS: 7778-77-0) acquired from Fluka. D(+) saccharose extra pure (16104, 99%, CAS: 57-50-1); sodium chloride (NaCl) (puriss p.a., CAS: 7647-14-5); sodium hydroxide (NaOH) (purified, CAS: 1310-73-2) obtained from Riedel-de-Haën (Germany).

5.2.2 Diclofenac Exposure Assay (250 $ng.L^{-1}$)

Mussels *M. galloprovincialis* (n = 245, average shell length size: 60 ± 3 mm, width: 34 ± 2 mm) were collected from Ria Formosa Lagoon (Portugal) and transported alive to the laboratory. After shell-cleaning mussels were placed in separate aquaria acclimatizing during 7 days (n = 35, 1 $mussel.L^{-1}$). Four aquaria were set as controls and three aquaria set for two-week exposure to 250 $ng.L^{-1}$ DCF where at every 48 hours seawater was changed and DCF concentration re-established. Throughout the full duration of the experiment, all aquaria were kept at temperature (19.8ºC ± 1.6), salinity (34.8 ± 0.2), pH (7.8 ± 0.1) and oxygen saturation (97% ± 4.8).

At each set up time (0, 3, 7, and 15 days), mussels (n = 20) were collected from control and exposure aquaria and subjected to individual shell biometric data measurement (length, width) and tissue dissection (gills, digestive gland and gonads). Single tissue samples were immediately frozen in liquid nitrogen and stored at -80ºC prior to biomarker analysis. For condition index (CI) assay, 15 mussels of each aquarium were individually weighted regarding the ratio:

$$CI = \frac{\text{whole soft tissue (wet weight)}}{\text{whole body tissue w/shell (wet weight)}} \text{ x } 100$$

5.2.3 Antioxidant Enzymes Analysis

Each gills (n = 5) and digestive gland (n = 5) tissue sample was individually homogenized on ice with 20 mM TRIS buffer pH 7.6 (1 mM of EDTA + 0.5 M of saccharose + 0.15 M of KCl + 1 mM of DTT). Homogenates were centrifuged at 500 × *g* for 15 minutes (4ºC) and supernatants recentrifuged at 12,000 × *g* for 45 minutes (4ºC). Resulting cytosolic fraction volumes were measured and a 100 µl aliquot of each sample was collected for total protein quantification (Bradford, 1976). Samples were later purified applying Sephadex® G-25 gel columns for the removal of low molecular weight proteins.

Individual purified aliquots were analyzed (in triplicate) for each antioxidant enzyme activity spectrophometric assay, as following:

SOD activity was determined by the 50%-reduction of cytochrome *c* absorbance generated by xanthine oxidase/hypoxanthine system at 550 nm (McCord and Fridovich, 1969). SOD activity is expressed as arbitrary units (U).mg^{-1}total protein.min^{-1}.

CAT activity was assessed by the absorbance decrease due to hydrogen peroxide (H_2O_2) consumption at 240 nm (Greenwald, 1985). CAT activity is expressed as µmol.mg^{-1} total protein.min^{-1}.

GR activity was determined by the increase of co-factor NADPH oxidation using oxidized glutathione (GSSG) as substrate at 340 nm (adaptation from Cribb *et al.*, 1989). GR activity is expressed as µmol NADPH oxidized.mg^{-1} total protein.min^{-1}.

GST activity was performed following the conjugation of reduced glutathione (GSH) with 1-chloro-2,4- dinitrobenzene (CDNB) at 340 nm (adaptation from Habig *et al.*, 1974). GST activity is expressed as µmol of CDNB conjugate formed.mg $^{-1}$ total protein.min^{-1}.

5.2.4 LPO Analysis

Gills (n = 10) and digestive gland (n = 10) tissue samples were individually homogenized with 20 mM TRIS-HCl buffer (pH 8.6) and butylated hydroxytoluene (BHT) in a 100:1 µl ratio on ice. Homogenates were centrifuged at 30,000 × *g* for 45 minutes (4ºC) to precipitate cytosolic fraction. An aliquot was saved for total protein quantification (Bradford, 1976) and remaining cytosolic fraction was used to quantify

the formation of LPO by-products malondialdehyde (MDA) and (2E)-4-hydroxy-2-nonenal (HNE) at 586 nm (adaptation from Erdelmeier *et al.*, 1998). LPO levels are expressed as µmol MDA.g^{-1}total protein.

5.2.5 AChE Analysis

Gills (n = 5) were homogenized individually in 100 mM Tris-HCl buffer (pH 8.0) and 100:1 µl of Triton on ice. Homogenates were centrifuged at 12,000 ×*g* for 30 minutes (4ºC). Resulting supernants were separated in aliquots for total protein determination (Bradford, 1976) and AChE activity analysis following Ellman *et al.* (1961). AChE activity was determined measuring the increase of 5-mercapto-2-nitrobenzoate formation (yellow color) resultant from the reaction of thiocholine (substrate) with DTNB at 405 nm (ε = 13.6 $mM^{-1}.cm^{-1}$). AChE activity is expressed nmol.mg^{-1} total protein.min^{-1}.

5.2.6 ALP Analysis

Sex-differentiated gonads (n = 10) were individually homogenized in 25 mM Hepes–NaOH buffer (pH 7.4) (125 mM NaCl + 1 mM DTT + 1 mM EDTA) on ice (adaptation from Blaise *et al.* (1999)). Homogenates were centrifuged at 12,000 × *g* for 30 minutes (2ºC) and pellets discarded. A supernant aliquot was reserved to determine total protein content (Bradford, 1976). Remaining cytosolic fractions were adjusted with 35% acetone and centrifuged at 10,000 ×*g* for 5 minutes. Pellets were later dissolved with 1 M NaOH and subjected to a 60ºC heating bath for 30 minutes. Phosphomolybdenum method was applied to quantify inorganic phosphate (KH_2PO_4) concentration at 660 nm (Stanton, 1968). ALP concentration is expressed as µg [PO_4].mg^{-1} total protein.

5.2.7 Statistical Analysis

Results are presented as mean ± standard deviation at each set of time. Two-way ANOVA was performed using SIGMAPLOT® to test differences between control and DCF exposed mussels in each tissue at each set of time. Holm-Sidak test was applied on biomarker difference discrimination within and when applicable between tissues through time. Pearson correlations between biomarkers were performed using XLSTAT® 2010 to verify each biomarker dependency. PCA were accomplished using XLSTAT® 2010 to assess the variability associated to each biomarker factorial weight

at each set of time and treatment group, for gills and digestive gland individually as well for gills vs. digestive glands. ALP levels in gonads were not considered in PCAs and AChE activities were only applied in gills PCA. Statistical significance was defined at $p < 0.05$ level.

5.3 Results

5.3.1 Condition index

No significant differences were found regarding CI (%) between controls and DCF-exposed mussels throughout the duration of the experiment (min: 13.8% ± 2.1, max: 15.8% ± 3.0) ($p > 0.05$).

5.3.2 Antioxidant Enzymes

Generally, antioxidant enzyme basal activities were higher in gills than in digestive glands, with the exception of CAT activity which were similar in both tissues.

SOD activity was stable in controls throughout the experiment (Figures 5.1A and B). DCF-exposed gills showed a significant 2.5-fold higher SOD activity on the 3rd day ($p < 0.05$) while for DCF-exposed digestive gland the increment was less pronounced and only significant after the first week (2.1-fold higher than control). Furthermore, both tissues exhibited a progressive SOD activity inhibition by the end of the experiment, being only significantly lower than controls in gills ($p < 0.05$). In gills, CAT activity showed no significant differences within controls and between exposed gills, although average values were lower in the later ones ($p > 0.05$). In digestive gland, even though controls exhibited a slight but not significant fluctuation ($p > 0.05$) exposed mussels clearly showed an increase in CAT activity after the 3rd day (1.5-fold higher than controls), progressively declining to lower levels than controls by the end of exposure. This enzyme is positive related with SOD activity in gills ($r = 0.90$, $p < 0.05$ in Table 5.3). Likewise, GR activity fluctuation in gills showed no differences in the controls ($p > 0.05$) and in DCF exposed mussels being positively related with both SOD in gills and also CAT in digestive gland ($r = 0.984$ and $r = 0.902$, $p < 0.05$ respectively; Table 5.3). These relationships are due to the concomitant 3.1-fold increase of GR activity in exposed gills on the 3rd day and following decrease to control activities after two weeks. Furthermore in digestive gland, even though GR activities varied within controls, the

equally progressive increase of GR activity from the beginning of the exposure until the first week followed by a decrease to lower values than controls after two weeks ($p < 0.05$) explains the direct relationship between GR and SOD activities in this tissue ($r = 0.817$, $p < 0.05$; Table 5.3). Phase II GST activities decreased overtime in control' gills, and after the first week in digestive glands ($p < 0.05$). In exposed-tissues GST activity was generally higher but not different from controls, except for day 7. Nevertheless, unlike any other enzymes, GST activities was directly related between tissues ($r = 0.925$, $p < 0.05$; Table 5.3) and with SOD activity in digestive gland ($r = 0.793$ with gills and $r = 0.843$ with digestive glands, Table 5.3).

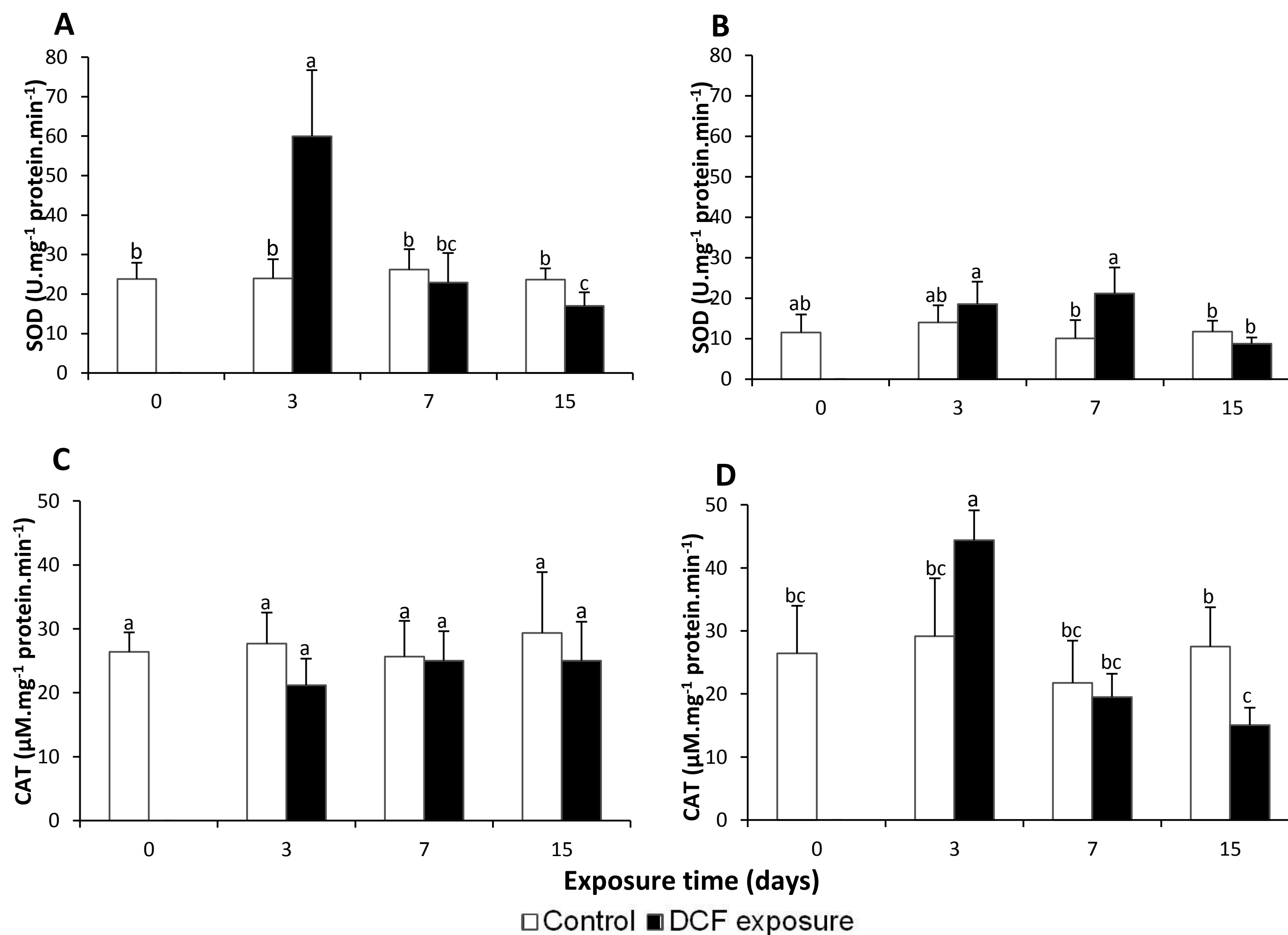

Figure 5.1: Antioxidant enzyme activities (mean ± standard deviation) in controls and DCF exposed *M. galloprovincialis* tissues. SOD activity in gills (A) and digestive gland (B); CAT activity in gills (C) and digestive gland (D); GR activity in gills (E) and digestive gland (F); GST activity in gills (G) and digestive gland (H). Different letters express significant differences ($p < 0.05$).

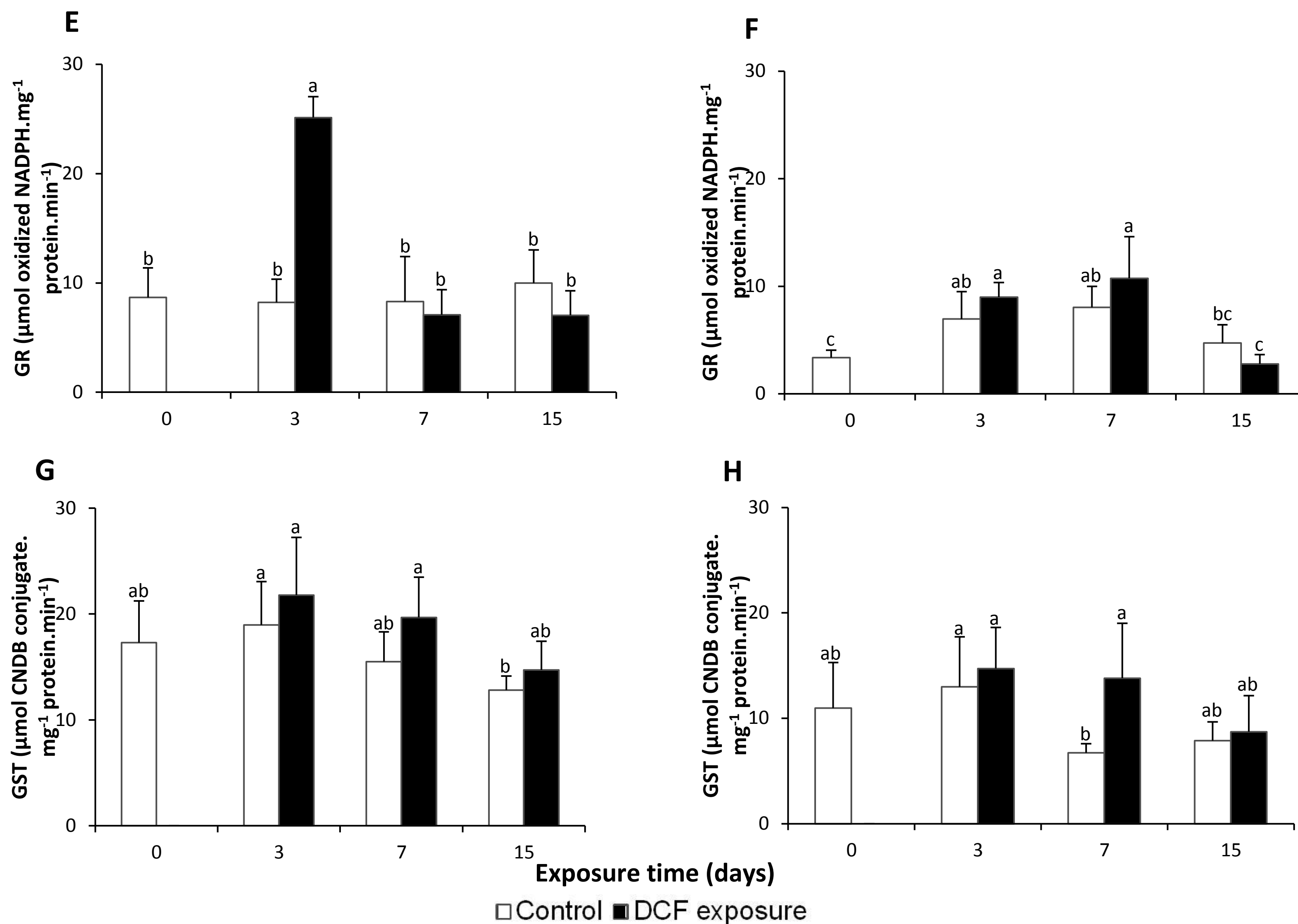

Figure 5.1 (Continuation): Antioxidant enzyme activities (mean ± standard deviation) in controls and DCF exposed *M. galloprovincialis* tissues. GR activity in gills (E) and digestive gland (F); GST activity in gills (G) and digestive gland (H). Different letters express significant differences ($p < 0.05$).

Table 5.3: Pearson correlations between biomarkers in gills (G), digestive gland (DG) and sex-differentiated gonads (F or M)

Variables	SOD G	SOD GD	CAT G	CAT DG	GR G	GR DG	GST G	GST DG	LPO G	LPO DG	AChE	ALP F	ALP M
SOD G	-												
SOD GD	0.495	-											
CAT G	-0.733	-0.447	-										
CAT DG	**0.902**	0.415	-0.410	-									
GR G	**0.984**	0.421	-0.697	**0.903**	-								
GR DG	0.450	**0.817**	-0.420	0.296	0.318	-							
GST G	0.657	**0.793**	-0.689	0.577	0.569	0.656	-						
GST DG	0.534	**0.843**	-0.505	0.539	0.489	0.527	**0.925**	-					
LPO G	0.711	0.166	**-0.801**	0.553	0.738	0.073	0.577	0.475	-				
LPO DG	**0.831**	0.005	-0.572	0.731	**0.889**	0.024	0.201	0.091	0.738	-			
AChE	0.278	**0.871**	-0.457	0.071	0.244	0.656	0.524	0.617	0.079	-0.070	-		
ALP F	0.027	0.507	-0.222	-0.143	0.089	0.173	0.083	0.304	0.012	-0.038	**0.827**	-	
ALP M	-0.007	-0.445	-0.204	-0.286	0.060	-0.231	-0.546	-0.655	0.093	0.401	-0.073	0.216	-

Bold values are different from zero at $p < 0.05$.

5.3.3 LPO

LPO levels were generally higher in gills than in digestive gland (Figures 5.2A and B). In gills, controls showed a significant decrease from the first week to the second, although not different from the remaining controls. In exposed gills, LPO levels were quite transient, with higher levels found on the 3rd and 15th day (not different from respective controls) interposed by a significant 2.3-fold decrease after the first week. Additionally, LPO levels in gills are inversely related to CAT activities (r = -0.801, $p <$ 0.05; Table 5.3). In digestive glands, LPO levels progressively increased in controls even if only significantly at the beginning of the experiment ($p < 0.05$), while in DCF-exposed ones alike for gills, the pattern oscillated from a significant 2.4-fold increase on the 3rd day to a 2.9-fold decrease after the first week ($p < 0.05$), followed by a later increase to levels not different from controls by the end of the exposure treatment. Finally, LPO levels in digestive glands are directly related with both SOD and GR activities in gills (r = 0.831 and r = 0.889 respectively, $p < 0.05$; Table 5.3).

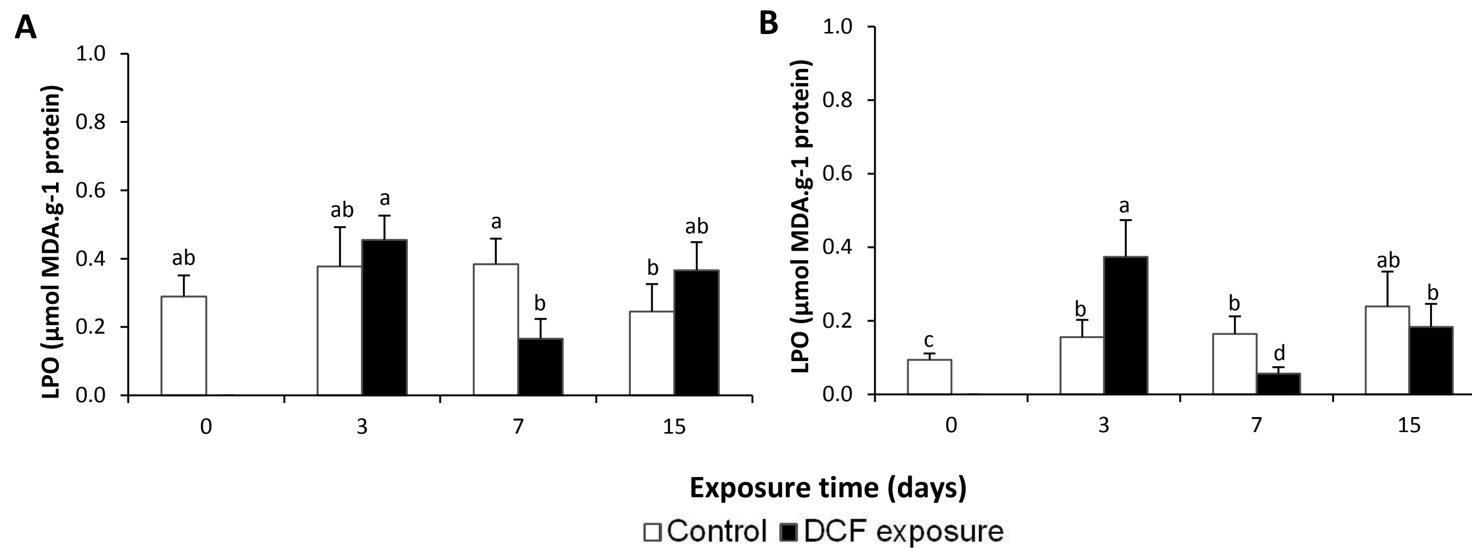

Figure 5.2: LPO (mean ± standard deviation) in mussels *M. galloprovincialis* control and DCF-exposed gills (A) and digestive glands (B) over 15 days. Different letters express significant differences ($p < 0.05$).

5.3.4 AChE

AChE activity in gills (Figure 5.3) did not vary within controls overtime ($p > 0.05$), while in exposed gills AChE activity gradually increased, reaching a significant higher levels after the first week (2.1-fold higher than control), and falling again to control levels by the end of the experiment. Furthermore, AChE activity was directly related with SOD activity in digestive gland and ALP levels in females-gonads ($r = 0.871$ and $r = 0.827$ respectively, $p < 0.05$; Table 5.3).

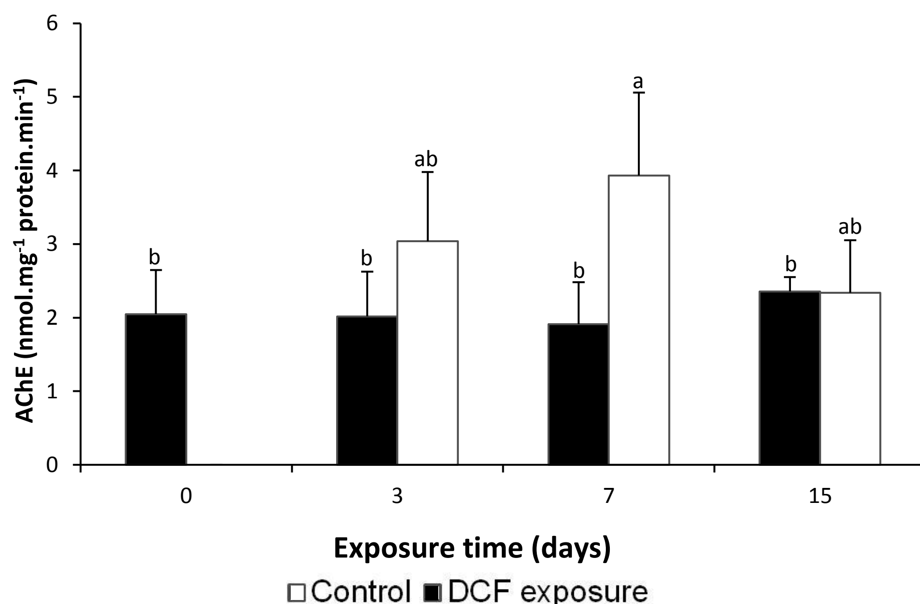

Figure 5.3: AChE activity (mean ± standard deviation) in control and exposed mussels *M. galloprovincialis* gills to 250 ng.L^{-1} of DCF during 15 days. Different letters express significant differences ($p < 0.05$).

5.3.5 ALP

ALP levels (Figure 5.4) varied significantly mostly in female gonads compared to males'. In female-controls ALP levels significantly decreased over the first week (overall min: 390 ± 116 µg $PO_4.mg^{-1}$ protein) followed by a significant increase at the end of the experiment whereas exposed-female gonads exhibited an inverse evolution pattern in which ALP levels progressively increased over the 3rd day (1.9-fold higher than control) and first week reaching the maximum: 1284 ± 212 µg $PO_4.mg^{-1}$protein approximately 3.3-fold higher than controls ($p < 0.05$). In exposed-males ALP levels were more consistent throughout time than in females, being 1.5-fold higher than control on the 3rd day ($p < 0.05$) which in turn was lower than remaining controls ($p < 0.05$). Finally, except for the first week, no other differences were observed between exposed males and females which increased significantly over time.

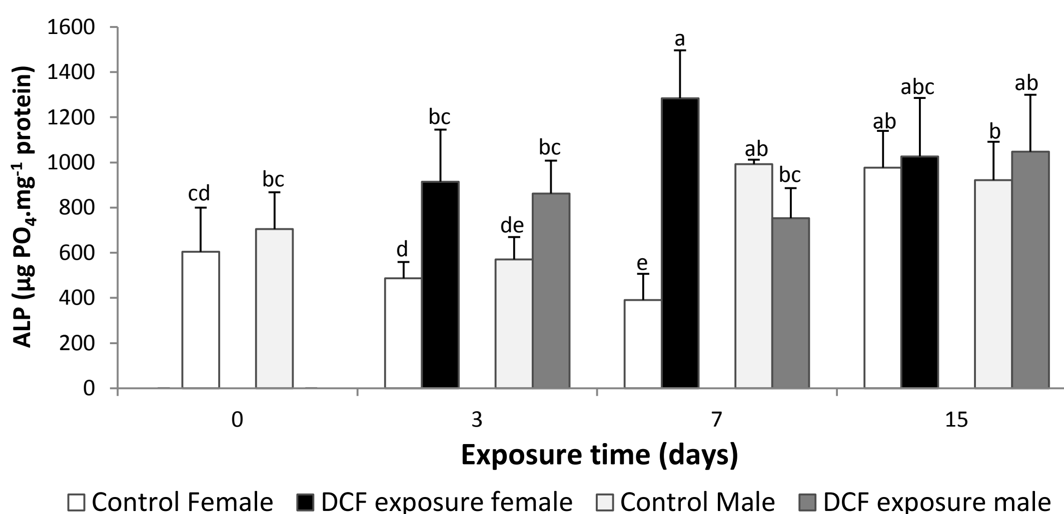

Figure 5.4: ALP levels (mean ± standard deviation) in control and exposed mussels *M. galloprovincialis* sex-differentiated gonads to 250 ng.L^{-1} of DCF during 15 days. Different letters express significant differences (p < 0.05).

5.3.6 PCA in Gills and Digestive Gland

In the gills (Figure 5.5A) PCA represents 81% of total variance (TVar). The first component (PC1) explains approximately 52% of the TVar, giving evidence to the separation between controls and DCF-exposed gills by the antagonist distribution of CAT activity in relation to the remaining enzymes activities and particularly negatively related to LPO levels (see Table 5.3). Two aspects are underlined in PC1: day 3 DCF-exposed gills are more distant from controls due to significant higher and positively related SOD and GR activities (see Table 5.3) and LPO levels, while the opposing lower CAT activity explain day 15-exposed gills clustering behavior with controls. The second component explains 29% of the TVar showing CI, CAT, GR and SOD activities and LPO divergent distribution compared to AChE and GST activities. AChE activity induction is the major influencing variable on day 7 DCF-exposed gills dispersal.

In digestive gland, PCA (Figure 5.5B) represents almost 85% of TVar, showing a more balanced influence of both components: PC1 (48%) and PC2 (37%) of TVar. AChE activity was not included as it was only measured in gills. PC1 clearly clusters most controls and particularly day 15-exposed digestive gland opposing CI to overall biomarkers responses. Moreover and particularly highlighted by the PC2, while day 3 DCF-exposed digestive gland was further influenced by higher levels of LPO and CAT activity, day 7 DCF-exposed digestive gland dispersal was associated by the positive relationship between SOD, GST and GR activities combined to a significantly lower LPO level.

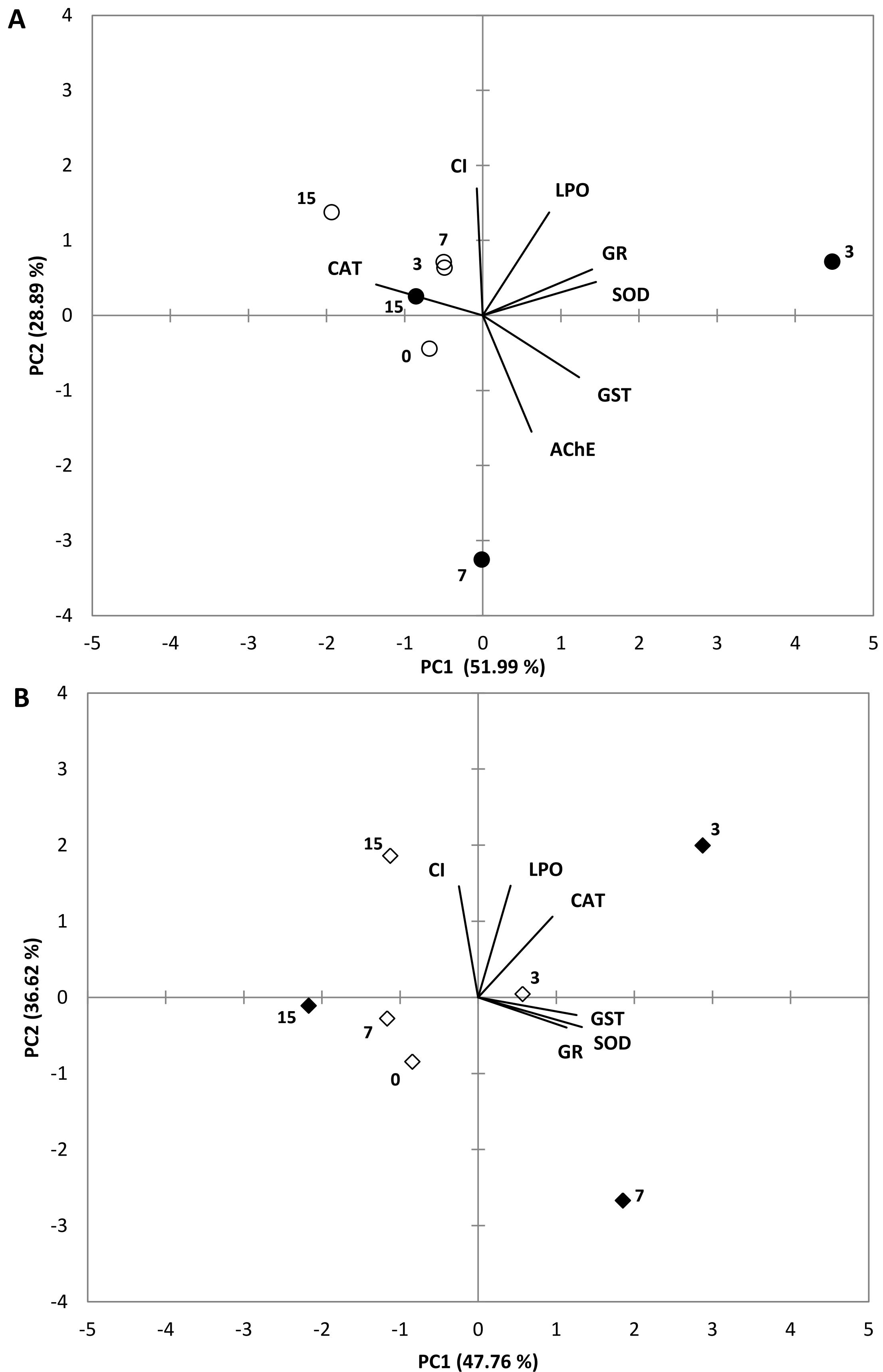

Figure 5.5: PCA concerning (A) SOD, CAT, GR, GST, AChE activities, LPO levels and CI on 81% of total variance in *M. galloprovincialis* gills (○– gills controls, ● – DCF exposed gills) - (B) SOD, CAT, GR, GST activities, LPO levels and CI on 85% of total variance in digestive gland (♦ – digestive gland controls, ◊ – DCF exposed digestive gland) between groups over exposure time (days: 0, 3, 7 and 15)

PCA (Figure 5.6) (82% of TVar) represents a tissue-specific integration between antioxidant enzyme activities and LPO levels in which most variance is represented by PC1 (61% of Tvar) and PC2 only explains 22%. This PCA gives evidence 1) to the separation between tissues, as gills (non and DCF-exposed) exhibit higher activities of antioxidant enzymes and LPO than digestive glands (except CAT on day 3 DCF-exposure), 2) to an overall clustering of both tissues controls in relation to most exposed treatment groups with the exception of 15 DCF-exposed gills which in turn give evidence to an antioxidant system recovery in this tissue, 3) the higher effect of DCF exposure over the antioxidant system in both tissues on the 3rd day due to higher SOD and GR activities (in gills) and the simultaneous enhancement of CAT activity and LPO levels (in digestive glands).

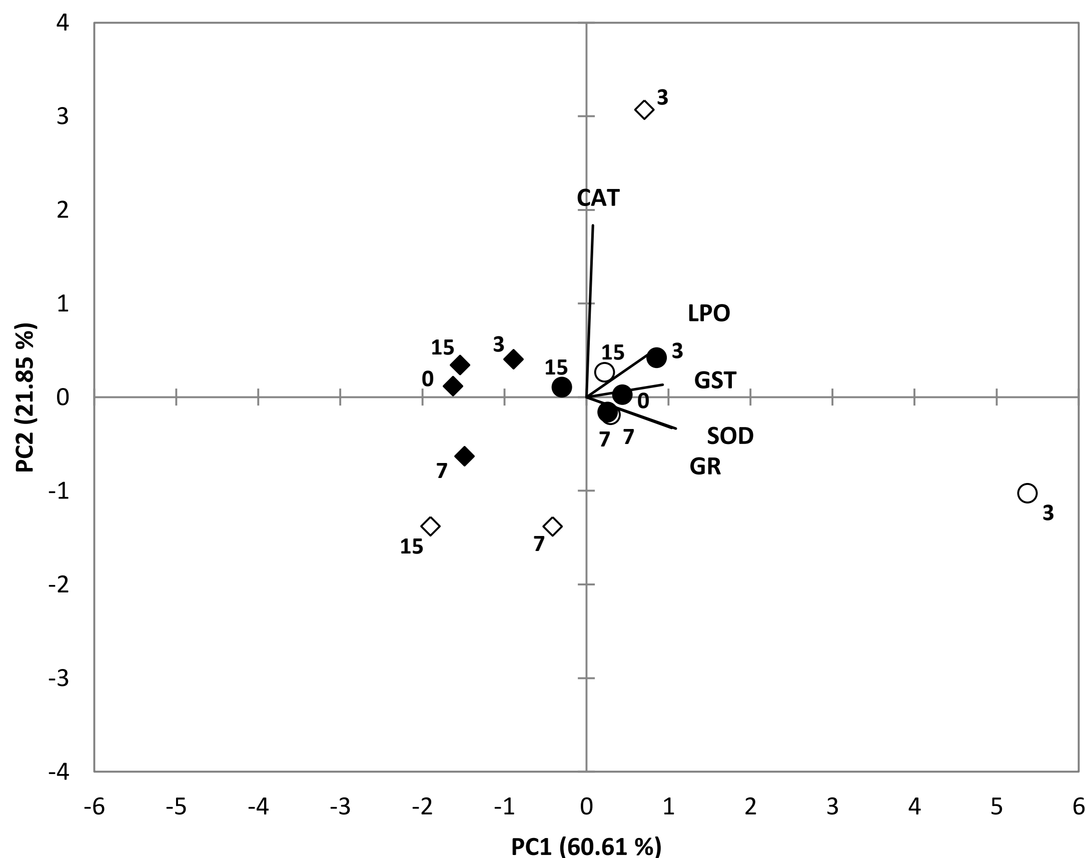

Figure 5.6: PCA representing 82% of total variance of tissue-specific integration of antioxidant enzyme activities (SOD, CAT, GR and GST) and LPO levels between controls and DCF exposed mussels *M. galloprovincialis* (● – gills control, ○ – DCF exposed-gills, ♦– digestive gland controls and ◊ – DCF exposed-digestive gland) between groups over exposure time (days: 0, 3, 7 and 15)

5.4 Discussion

This study confirms that even not significantly affected on its general physiological condition, mussels' exhibit significant biomarker responses when continuously exposed to an environmental realistic concentration of DCF (250 $ng.L^{-1}$) during two weeks.

5.4.1 Oxidative Stress

Invertebrates, like mammals, possess an inherent antioxidant defense system which prevents reactive oxygen radicals (ROS) derived-effects such as oxidative stress, LPO and apoptosis through the action of either non-enzymatic scavengers (e.g. glutathione-GSH), specific antioxidant enzymes (like, SOD, CAT and GR) and Phase II biotransformation enzyme GST which enables detoxification converting parental compounds into more extractable hydrophilic metabolites through its conjugation with reduced GSH (Viarengo *et al.*, 1995; Halling-Sorensen *et al.*, 1998; Van der Oost *et al.*, 2003).

Although DCF biotransformation after entering the body in aquatic organisms is yet unknown (Oviedo-Gómez *et al.*, 2010), several studies report the ability of NSAIDs to cause oxidative stress in aquatic species (Oviedo-Gómez *et al.*, 2010; Quinn *et al.*, 2011; Schmidt *et al.*, 2011; Gonzalez-Rey and Bebianno, 2011; 2012 - Chapters 3A -B). In mammals oxidative stress production by DFC is both linked to CYP-mediated DCF-bioactivation as well as *p*-quinone imines (derived from hydroxylated metabolites) involvement in redox cycling (Boesterli, 2003). Furthermore, NSAIDs inhibits COX pathway and consequently prostaglandins synthesis which is also associated to enhance basal ROS production via AA accumulation (Ardaillou *et al.*, 1987; Cocco *et al.*, 1999; Delaporte *et al.*, 2006).

Nevertheless, in this study antioxidant responses toward DCF exposure were quite time-specific and transient, in which the results showed a clearer enhancement of antioxidant activities in gills than in digestive glands (Figures 5.1 and 5.6) except for CAT activity. Only over the 3rd day a positive induction was shown regarding SOD and GR activities in gills and CAT activity in digestive glands (Table 5.3, Figure 5.6). These results indicate that antioxidant system is readily triggered in gills by the enhancement of SOD activity which is called to convert short-term DCF-derived ROS (such as super anion radical - $O_2^{-\bullet}$) into to less toxic molecules (like H_2O_2), which potentiates concomitant

CAT activity up regulation in digestive glands conversion of excess H_2O_2 into H_2O and O_2 (Viarengo *et al.*, 1995; Van der Oost *et al.*, 2003). At the same time the increase of gills-GR activity reveals the need of an increased conversion of oxidized glutathione (GSSG) into reduced GSH which in higher concentrations will act as a direct ROS scavenger and cofactor in this tissue cytosol (Viarengo *et al.*, 1995). Moreover, NSAID radicals in mammals can oxidize GSH and NADPH based on peroxidase-catalyzed production (Boelsterli, 2003). Even so, in this study the activity of GST was not altered significantly in exposed tissues (Figure 5.1G and H) and showed to be directly related with digestive gland-SOD rather than with GR or CAT activity (Table 5.3). GST and CAT activities were reported to be directly related to *M. galloprovincialis* digestive glands exposed to antilipidemic bezafibrate, anticonvulsant carbamazepine, β-blocker propranolol and NSAID ibuprofen (Gonzalez-Rey and Bebianno, 2012 - Chapter 3B; Canesi *et al.*, 2007; Martín-Díaz *et al.*, 2009; Franzellitti *et al.*, 2011). However, in bivalves *D. polymorpha* and *Mytilus spp.* exposed to a higher concentration of DCF (1 $\mu g.L^{-1}$) GST was inducted along with LPO increase in visceral mass and digestive gland (Quinn *et al.*, 2011; Schmidt *et al.*, 2011) which confirms that at this concentration DCF does not promote detoxification derived from Phase II conjugation with available reduced GSH.

In addition, DFC exposure only induced clear LPO levels in mussels' digestive gland over the 3rd day which upturn was positively related with SOD and GR activities in gills highlighting evidences of transport between tissues related to its specific metabolic functionality. The significant LPO decrease afterwards attests those enzyme activities parallel effectiveness on the counteraction of ROS favoring the recovery of antioxidant system in this tissue. Whereas in exposed gills, LPO levels were generally not different from controls, showing an effective LPO decrease on the 7th day which was further associated to its negative relationship with the overall inhibitory tendency of CAT activity ($p > 0.05$). NSAID ibuprofen exposed mussels also exhibited higher LPO in digestive gland directly related with GR activity, while in gills a much higher LPO level was directly associated to SOD activity enhancement with concomitant inhibition of CAT, GR and GST activities. Even though, the mussel exposure to NSAIDs ibuprofen and DCF promoted a different set of tissue-specific antioxidant responses over the first days, both experiments showed an overall recovery of tissues by the end of the experiment (Gonzalez-Rey and Bebianno, 2011; 2012 - Chapter 3).

5.4.2 Neurotoxic Effect and Endocrine Disruption

AChE activity is critical in neuromuscular system functions (such as muscular contraction) as the promoter of neurotransmitter acetylcholine (ACh) hydrolysis to choline and acetic acid. AChE-inhibition is considered as a biomarker of exposure to several contaminants such as organophosphorus pesticides, detergents and APIs (Ricciardi *et al.*, 2006; Matozzo and Marin, 2005; Solé *et al.*, 2010). However, in this study DCF clearly induced AChE activity in gills during the first week, which is only in agreement with the results of an exposure of the same species to FLX (75 $ng.L^{-1}$) (Gonzalez-Rey and Bebianno, *in press* - Chapter 4) whereas AChE activity was inhibited by other APIs paracetamol (23 and 403 $\mu g.L^{-1}$) in *M. galloprovincialis* gills (Solé *et al.*, 2010) and diazepam (4, 20 and 100 nmol per mussel) in *E. complanata* visceral mass (Gagné *et al.*, 2011). AChE induction has been associated to cell apoptosis in various types of human and mammals cells being hypothesized that AChE is released after cell membrane disruption (Zhang *et al.*, 2002). Also a link between estrogenic and AChE activity was reported in mice brain cells, in which an enhancement of AChE was observed when isoflavones (phytoestrogens) bind to the estrogen receptor (Isoda *et al.*, 2002; Mun'im *et al.*, 2004) and in the presence of low levels of estradiol (Tsuchiya *et al.*, 2004).

Vitellogenins (Vtg) are naturally synthesized in females and inactive in males, the increase of ALP concentration as result of Vtg alkali hydrolysis is considered a sign of endocrine disruption (ED) by estrogenic compounds (like pesticides, detergents, pharmaceuticals, industrial chemicals, etc.) particularly in males (Blaise *et al.*, 1999; Gagné *et al.*, 2002; Porte *et al.*, 2006; Matozzo *et al.*, 2008). In this study, the induction of ALP levels was more evident in DCF-exposed females than males (Figure 5.4). Even though, the concomitant increase of ALP levels in controls, particularly in males may have overshadowed the significance of DFC exposure in sex-differentiated gonads. In *M. galloprovincialis* exposed to 250 $ng.L^{-1}$ NSAID IBU and 75 $ng.L^{-1}$ antidepressant FLX shown respectively much stronger ALP levels enhancement and general ALP levels depletion in exposed-gonads (Gonzalez-Rey and Bebianno, 2012; 2013 – Chapter 3 and 4).

In this sense, the direct relationship between ALP levels in females and AChE (Table 5.3) give evidence to an unknown DFC-derived interference with mussels' estrogen receptors rather than inducing neurotoxic effects just due to ACh accumulation. However this outcome can only be complimented by verifying the estradiol and prostaglandins levels in mussels' tissues.

Additionally, unlike the unaltered ALP levels observed after the exposure of mussels *D. polymorpha* and *Mytilus spp* to a higher concentration of DCF (1 $\mu g.L^{-1}$) (Quinn *et al.*, 2011; Schmidt *et al.*, 2011) the present results showed some evidences of endocrine disruption at a more environmentally relevant concentration especially in females.

5.5 Conclusions

In conclusion, mussels exposed to DCF were affected mostly during the first days showing later overall recovery responses. The antioxidant system was swiftly triggered particularly in gills and evidence of damage was observed in digestive glands. Likewise, DCF significantly induced a direct relationship between AChE activity and ALP levels in females . Finally, the enhancement of AChE does not give evidence to the breakdown of cholinergic neurotransmission functions but rather to potential estrogenic activity alteration or cell apoptosis, nevertheless further assessment concerning estradiol and prostaglandin content in mussel tissues would greatly clarify this outcome.

5.6 References

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Chapter 6

Effects of APIs mixtures in mussel *M. galloprovincialis*

6 *Effect of APIs mixtures in mussel* M. galloprovincialis *through a multibiomarker approach*

Abstract

Active pharmaceutical ingredients (APIs) are considered environmental emergent contaminants. APIs occurrence in surface water has been widely reported resulting from incomplete waste water treatment plants (WWTPs) removal processes and improper disposal. The assessment of potential effects in non-target organisms is still very scarce. Mussels *Mytilus galloprovincialis* were exposed to single environmental relevant concentrations of 250 ng.L^{-1} ibuprofen (IBU), 250 ng.L^{-1} diclofenac (DCF) and 75 ng.L^{-1} fluoxetine (FLX) and as mixtures without (MIX 1) and with 5 µg.L^{-1} copper (Cu) (MIX 2) during two weeks. A multibiomarker approach was applied to assess APIs-derived responses in exposed mussels: i) condition index (CI); ii) superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and phase II biotransformation glutathione-S-transferase (GST) enzymatic activities and lipid peroxidation levels (LPO) associated to oxidative stress; iii) acetylcholinesterase (AChE) activity related to neurotoxic effect and iv) vitellogenin-like proteins by alkali-labile phosphate (ALP) method in sex-differentiated gonads linked to endocrine disruption. APIs mixtures induced transient antioxidant enzymes activity patterns (particularly MIX 1) which were not enough to prevent LPO damage particularly in MIX 2 exposed gills. Moreover, neither one of the mixtures altered AChE activity alterations, while both enhanced vitellogenin in females over first week. Finally, this study confirms variable tissue and time specific biomarkers susceptibility of *M. galloprovincialis* to single and APIs mixtures at environmental relevant concentrations and several interactions between each mixture component on each biomarker outcome.

6.1 Introduction

All APIs' therapeutic classes have been detected in surface waters (Kümmerer *et al.*, 2009; Santos *et al.*, 2010) (see previous chapters, and particularly Chapter 2). In an ecotoxicological viewpoint it is essential to consider the interactions of multiple stressors and associated effects regarding biochemical responses alterations of exposed organisms since they may differ entirely from each contaminant singular mode of action. Therefore the assessment of APIs mixtures exposure effects is particularly relevant since APIs are biological active compounds. Multistressor ecotoxicity assessment is very complex relaying on the assumption of a concerted action between contaminants whether chemicals act on an independent non-interactive manner ("simple similar action", "dose addition" or "independent action") or in an interactive one producing combined effects which can be synergetic or antagonistic departed from the additivity between individual components (EPA, 2002; IPCS, 2009). Regardless of the increasing research effort focused on the assessment of potential effects of APIs exposure in non-target organisms in which most focus in single APIs exposure (see Kümmerer *et al.*, 2009; Santos *et al.*, 2011 – Chapter 3-5) to our knowledge there are very few studies addressing the effects of either binary or multiple pharmaceutical mixtures exposure in aquatic organisms (as single species or as microcosms studies) (Table 6.1).

As mentioned previously, the selected NSAIDs IBU and DCF and SSRI FLX are amongst the most frequently detected in surface waters such reaching up to 2.4 $\mu g.L^{-1}$, 15 $\mu g.L^{-1}$ and 0.6 $\mu g.L^{-1}$ respectively (Jux *et al.*, 2002; Pailler *et al.*, 2009; Benotti and Brownawell, 2008) and are associated to several deleterious biological end-points in mussel species (see Chapters 3-5) regarding oxidative stress and DNA damage induction, physiological (e.g. growth, swimming/mobility, feeding and territorial behavior decrease), cytotoxicological (e.g. cell viability decrease) and reproductive fitness (e.g. vitellogenesis, embryonic development and spawning induction) alterations. Even though, it is absolutely unreal to mimic a true environmental scenario through laboratory conditions it is essential to address the potential exposure effects derived from different types of APIs mixtures using a species proven to be extremely vulnerable to APIs discharges such as mussel *M. galloprovincialis* testing possible synergistic or antagonistic interactions between APIs and between APIs with other commonly

detected aquatic contaminants such as metals and in particular copper (Cu) (Chapter 1). The latter despite of being an essential metal and naturally occurring in ranges between 0.20 to 30 μg.L^{-1}, up to 200 mg.L^{-1} in surface waters adjacent to mining areas (EPA, 2007), was reported to promote ROS-generation (mostly by its involvement in Fenton-type redox reactions) with subsequent toxic effects induction in biomolecules, namely enzymatic activities alteration and LPO (Pipe *et al.*, 1999; Dondero *et al.*, 2006; Company *et al.*, 2004; Funes *et al.*, 2006; Maria and Bebianno, 2011).

In this sense, the aim of this Chapter focuses on the effects of two mixtures of multiple stressors: one induced by a mixture od the APIS used on the previous chapters and the other using the same mixture od emerging contaminants (APIs) combined with a classical contaminant (Cu) and compare the effects on mussel *M. galloprovincialis* single APIs exposure (Gonzalez-Rey and Bebianno, 2011; 2012; 2013 – see preceding Chapters 3-5). Mussels were exposed to two mixtures consisting one of 250 ng.L^{-1} IBU + 250 ng.L^{-1} DCF + 75 ng.L^{-1} FLX (mixture 1) and the other of 250 ng.L^{-1} IBU + 250 ng.L^{-1} DCF + 75 ng.L^{-1} FLX + 5 μg.L^{-1} of Cu (mixture 2) for two weeks. The main goal was to compare the effects on biomarkers of susceptibility (antioxidant enzymes SOD, CAT, GR and GST), damage (LPO), neurotoxicity (AChE) and endocrine disruption in mussels' gills, digestive gland and in sex-differentiated gonads and compared them with previously obtained from each selected APIs single exposure.

Table 6.1: Effects of APIs mixtures in aquatic organisms

Species	API mixture	Concentration ($\mu g.L^{-1}$)	Test		Biological end-point	Effect	Reference
Algae							
Desmodesmus subspicatus	CLA, CAR, IBU-Na, DCF-Na, NAP-Na, CAP, METF, PRO and MET	1×10^2, 0.32×10^3, 3.2×10^3, 10×10^3, 32×10^3, 100×10^3, 320×10^3	acute and chronic	3 – 7 days	EC_{50} average growth rate	• EC_{50}: 5.8 $mg.L^{-1}$ (PRO) - 320 $mg.L^{-1}$ (MET and NAP)	Cleuvers *et al.*, 2003
	DCF, IBU, NAP and ASA	1×10^3, 3.2×10^3, 1×10^4, 32×10^3, 1×10^5, 3.2×10^5	acute	24, 48 hours	EC_{50} immobilization	• EC_{50} (72 - 626 $mg.L^{-1}$)	Cleuver *et al.*, 2004
Dunaliella tertiolecta	binary mixture: TRI with FLX and SIM with CLA	1.6, 4.9, 14.8, 44, 133 (TRI); 2.7, 8, 24, 72, 216 (FLX); 1560, 6250, 25×10^3, 1×10^5, 4×10^5 (SIM and CLA)	acute	0, 24, 48, 72, 96 hours	EC_{50} cell density	• cell density reduction and additive effect of mixtures	De Lorenzo and Flemming, 2008
Plantae							
Lemna minor	CLA, CAR, IBU-Na, DCF-Na, NAP-Na, CAP, METF, PRO and MET	1×10^2, 0.32×10^3, 3.2×10^3, 10×10^3, 32×10^3, 1×10^5, 3.2×10^5	acute and chronic	3 – 7 days	EC_{50} average growth rate	• EC_{50}: 7.5 $mg.L^{-1}$ (DCF) - > 320 $mg.L^{-1}$ (MET)	Cleuvers *et al.*, 2003
Crustacean							
Daphnia magna	CLA, CAR, IBU-Na, DCF-Na, NAP-Na, CAP, METF, PRO and MET	0.1×10^3, 0.32×10^3, 3.2×10^3, 10×10^3, 32×10^3, 1×10^5, 3.2×10^5	acute and chronic	48h	EC_{50} immobilization	• EC_{50} range: 7.5 $mg.L^{-1}$ (PRO) - 174 $mg.L^{-1}$ (NAP)	Cleuvers *et al.*, 2003
	FLX and CLA	36 (FLX), 10 or 100 (CLA)	acute and chronic	6 – 30 days	survival, growth and reproduction	• mortality and malformations	Flaherty and Dodson, 2005
	DCF, CAR, MET and EE2	1×10^{-4}, 0.36, 0.50, 1.2	chronic (multi-gerational)		age of first reproduction, body length of females and neonates and number of offspring	• age reduction of first reproduction and increased body length at first reproduction. Impairments not predicted by the response to single substances	Dietrich *et al.*, 2010
	DCF, IBU, NAP and ASA	1×10^3, 3.2×10^3, 10×10^3, 32×10^3, 100×10^3, 320×10^3	acute		EC_{50} average growth rate	• EC_{50} from 68 - 166 $mg.L^{-1}$	Cleuver *et al.*, 2004
Hyalella azteca	PAR, DCF, GEM, IBU, NAP, ASA and TRI	1×10^{-4} each	multi-generational		survival, mating, body size or reproduction	• sex ratio increase, 17% more males	Borgmann *et al.*, 2007

Table 6.1: (Continuation).

Cnidaria							
Hydra attenuata	BEZ, CAR, SULF, GEM, TRIM, SUL, NAP, NOV, OXY, IBU and CAF	(mother solution: 7.5 x 10^{-3}, 0.033, 0.046, 0.059, 0.063, 0.099, 0.217, 0.33, 0.44, 1.2, 22.2 (respectively) exposed to serial dilutions 0.1, 1, 10, 100, 1000, 10000x	acute and chronic	96 hours	LC_{50} and EC_{50} morphology, feeding, hydranth number and attachment	• significant decrease in morphology at 0.1, 10 and 100× and significant increase at 1000×. All parameters significantly reduced at 10000×	Quinn *et al.*, 2009
Bivalve							
Mytilus edulis trossulus	binary mixtures: DCF and PRO	100, 1000 of concentration ratios of PRO75/DCF25; PRO50/DCF50 and PRO25/DCF75	chronic	14 and 19 days	"scope for growth"	• lower "scope for growth" for PRO25/$DCF75_{1000}$ and PRO50/$DCF50_{1000}$ (synergetic effect)	Ericson *et al.*, 2010
Mytilus galloprovincialis	BEZ and GEM (molar ration 1:1)	0.01, 0.1, 1[4]	acute	24 hours	immune and digestive gland functions	• concentration-dependent lysosomal membrane destabilization and extracellular lysozyme release similar to single exposure	Canesi *et al.*, 2007
Fish							
Danio rerio (liver ZFL cells)	ATE, BEZ, CAR, IBU, RAN, SAL, CYC, HYD, FUR, CIP, LIN, OFL, SUL	environmental concentration levels	acute	72 hours	gene expression profiles	• inhibition of cells proliferation *in vitro*; transcriptional repression and several genes up regulation	Pomati *et al.,* 2007
Oncorhynchus mykiss (cell line RTL-W1)	Similar mixtures between NSAIDS (IBU, NAP, KET, DCF); antidepressants (FLX, PAX, FLU); fibrates (FEN, CLA, BEZ and GEM) and dissimilar mixtures (DCF, BEZ, FLU and musk ketone and galaxolide)(less toxic); (IBU, CLA, FLX and musk xylene and celestolide) (more toxic)	0.1, 0.5, 1, 2, 5[5]	acute		EC_{50} cell viability	• additive effect of mixture: 7 - 50 µM (antidepressants), 160-260 µM (NSAIDs), 20-380 µM (lipid regulators); combined toxicity higher than additive in dissimilar mixtures	Schnell *et al.*, 2009

[4] nmol/animal
[5] toxic units

Table 6.1: (Continuation).

Pimephales promelas	BUP, FLX, SER and VEN	2.2×10^{-3}, 28×10^{-3} (FLX); 1.3×10^{-3}, 22×10^{-3} (SER); 117×10^{-3}, 798×10^{-3} VEN); 50×10^{-3}, 466×10^{-3} (BUP)	chronic		reproductive anatomy, physiology, and adult behavior	• effects not carried over compared to single exposure nor become additive within antidepressant mixture.	Schultz *et al.*, 2011
Microcosm							
Lemna gibba and *Myriophyllum sibiricum*	ATO, PAR, CAF, SUL, CAR, LEV, SER and TRIM	0, 0.044, 0.608, 2.664, and 24.538[6]	acute and chronic	35 days	EC_{10}, EC_{25}, EC_{50} and phytoxicity	• concentration-dependent response and phytotoxic injury	Brain *et al.*, 2004
phytoplankton, zooplankton, macrophytes, bacteria species and fish *Lepomis gibbosus*	IBU, FLX and CIP	6×10^{3}, 10×10^{3} (low treatment); 6×10^{4}, 1×10^{5} (medium treatment); 6×10^{5}, 1×10^{6} (high treatment)	acute and chronic	35 days	mortality, diversity, abundance, growth	• mortality (*Lepomis gibbosis*); diversity decrease and abundance increase (phytoplancton and zooplancton), growth decrease (macrophytes), abundance change (bacteria) at HT and or/MT	Richards *et al.*, 2004
Zooplancton: Rotifera, Cladocera, Copepoda	FLX, FLU and SER	0, 91, 188, 424, 872[7]	acute and chronic	4, 35 days	abundance and species richness	• abundance decrease (Rotifera and Copepoda); species richness (Copepoda)	Laird *et al.*, 2007

[6] $\mu mol.L^{-1}$
[7] nM

6.2 Materials and Methods

6.2.1 Chemicals

All the applied chemicals were obtained as described: ibuprofen (2-(4-isobutylphenyl) propanoic acid) (I4883, ≥ 98% GC, CAS:15687-27-1); diclofenac sodium salt (D68990, CAS: 15307-79-6); R-(-) fluoxetine hydrochloride (F1678, > 98%, CAS: 114247-09-5); tetramethoxypropane (MDA) (108383, CAS: 102-52-3); 1-methyl-2-phenylindole (99%, CAS: 3558-24-5); 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (D8130, ≥ 98% TLC, CAS: 69-78-3); acetyl thiocholine iodide (ATC) (A5751, ≥ 98% TLC, CAS: 1866-15-5); bovine albumin serum (BSA) (A9418 > 98%, CAS: 9048-46-8); butylated hydroxytoluene (BHT) (B1378, ≥ 99.0% GC, CAS: 128-37-0); cytochrome *c* from equine heart (C7752, > 95%, CAS: 9007-43-6); diethylenetriaminepentaacetic acid dianhydride (DTPA) (D6148, CAS: 23911-26-4); ethylenediaminetetraacetic acid (EDTA) (ED, ≥ 99%, CAS: 60-00-4); Fiske and Subbarow Reducer (F5428); glutathione reductase (G3664, CAS: 9001-48-3); HEPES (H3375, > 99.5%, CAS: 7365-43-9); hydrogen peroxide solution (H1009, 30% w/w, CAS: 7722-84-1); hypoxanthine (H9377, > 99%, CAS: 68-94-0); L-glutathione oxidized (GSSG) (G4501, > 98%, CAS: 27025-41-8); L-glutathione reduced (GSH) (G4251, > 98%, CAS: 70-18-8); methanesulfonic acid (> 99.5%, CAS: 75-75-2); triton x-100 (X6878, CAS: 9002-93-1) xanthine oxidase from bovine milk (X1875, CAS: 9002-17-9); β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADPH) (N8129, ≥ 97%, CAS: 606-68-8) were purchased from Sigma-Aldrich (Germany). Protein-assay dye reagent concentrate (phosphoric acid CAS: 7664-38-2 + methanol CAS: 67-56-1) was obtained from Bio-Rad Laboratories, Inc. (USA). 1,4-dithiothreitol (DTT) (≥ 99%, CAS: 3483-12-3); acetonitrile (99.8%, CAS 75-05-8); methanol (99.9%, CAS 67-56-1); natriumazide (sodium azide) (106688, ≥ 99%, CAS: 26628-22-8); potassium chloride (KCl) (104936, 99.5%, CAS: 7447-40-7); trichloroacetic acid (TCA) (100807, CAS: 76-03-9); TRIS-(hydroxymethyl)-aminomethane (≥ 99%, CAS: 77-86-1) were acquired from Merck (Germany). 1-chloro-2,4,-dinitrobenzene (CDNB) (24440, ≥ 98.0% GC, CAS: 97-00-7) and molybdate reagent solution (puriss p.a.) and potassium dihydrogen phosphate (60218, 99.5%, CAS: 7778-77-0) acquired from Fluka. D(+) saccharose extra pure (16104, 99%, CAS: 57-50-1); sodium chloride (NaCl) (puriss p.a., CAS: 7647-14-5);

sodium hydroxide (NaOH) (purified, CAS: 1310-73-2) obtained from Riedel-de-Haën (Germany).

6.2.2 APIs Mixtures Exposure Assay

Mussels *M. galloprovincialis* (n = 300, average shell length size: 58 ± 4 mm, width: 33 ± 2 mm) were collected from Ria Formosa Lagoon (Portugal) and transported alive to the laboratory. Mussels were subjected to shell-cleaning and placed in aquaria acclimatizing during 7 days at a rate of 1 mussel.L^{-1}. Aquaria were set for controls and for two-week exposure to either a mixture of: 250 ng.L^{-1} IBU + 250 ng.L^{-1} DCF + 75 ng.L^{-1} FLX (mixture 1) or a mixture of: 250 ng.L^{-1} IBU + 250 ng.L^{-1} DCF + 75 ng.L^{-1} FLX + 5 µg.L^{-1} Cu (mixture 2). At every 48 hours natural seawater was changed and mixtures concentration re-established. All aquaria were kept at temperature (19.9ºC ± 1.6), salinity (34.8 ± 0.2), pH (7.8 ± 0.1) and oxygen saturation (96.5% ± 4.3).

After 0, 3, 7, and 15 days, mussels (n = 30) were collected from control and both mixtures exposure aquaria and subjected to individual shell biometric data measurement (length, width) preceding to tissue dissection (gills, digestive gland and gonads). Single tissue samples were immediately frozen in liquid nitrogen and stored at -80ºC prior to biomarker analysis.

For condition index (CI) assay, 15 mussels of each aquarium were individually weighted regarding the ratio:

$$CI\ (\%) = \frac{Whole\ soft\ tissue\ (w.w)}{whole\ body\ tissue\ with\ shell\ (w.w)} * 100$$

6.2.3 Antioxidant Enzymes Analysis

Single gills (n = 5) and digestive gland (n = 5) tissue samples were individually homogenized on ice with 20 mM TRIS buffer pH 7.6 (1 mM of EDTA + 0.5 M of saccharose + 0.15 M of KCl + 1 mM of DTT). Resulting homogenates were centrifuged at 500 $\times$ *g* for 15 minutes (4ºC) and supernatants recentrifuged at 12,000 $\times$ *g* for 45 minutes (4ºC). Cytosolic fraction volumes were measured reserving a 100 µl aliquot of each sample for total protein quantification analysis (Bradford, 1976) and purified applying Sephadex® G-25 gel columns to remove low molecular weight proteins.

Each purified aliquot was analyzed in triplicate concerning the following spectrophotometric assay:

SOD activity was determined at 550 nm resulting from the 50% reduction of cytochrome *c* absorbance generated by xanthine oxidase/hypoxanthine system according to McCord and Fridovich (1969) and expressed as arbitrary units (U).mg^{-1} total protein.min^{-1}.

CAT activity was assessed at 240 nm through the absorbance decrease resulting from hydrogen peroxide (H_2O_2) consumption according to Greenwald (1985) and expressed as µmol.mg^{-1} total protein.min^{-1}.

GR activity was determined at 340 nm as an outcome of the increase of co-factor NADPH oxidation using oxidized glutathione (GSSG) as substrate following an adaptation from Cribb *et al.* (1989) and expressed as µmol NADPH oxidized.mg^{-1} total protein.min^{-1}.

GST activity was assessed at 340 nm resulting from the conjugation of reduced glutathione (GSH) with 1-chloro-2,4- dinitrobenzene (CDNB) following an adaptation from Habig *et al.* (1974) and expressed as µmol of CDNB conjugate formed.mg^{-1} total protein.min^{-1}.

6.2.4 LPO Analysis

Gills (n = 10) and digestive gland (n = 10) tissue samples were homogenized separately on ice with 20 mM TRIS-HCl buffer (pH 8.6) and butylated hydroxytoluene (BHT) in a 100:1 µl ratio. In order to precipitate cytosolic fraction homogenates were centrifuged for 45 minutes (4ºC) at 30,000 × *g*. An aliquot was reserved for total protein quantification according to Bradford (1976). The quantification of LPO levels was assessed in triplicate in each remaining cytosolic fractions following the formation of the by-products malondialdehyde (MDA) and (2E)-4-hydroxy-2-nonenal (HNE) at 586 nm according to an adaptation from Erdelmeier *et al.* (1997) and expressed as µmol $MDA.g^{-1}$ total protein.

6.2.5 AChE Analysis

Gills (n = 5) were homogenized separately on ice with 100 mM Tris-HCl buffer (pH 8.0) and 100:1 µl of Triton. Resulting homogenates were centrifuged for 30 minutes (4ºC) at 12,000 ×*g*. An aliquot of the each supernant was saved for the analysis of total protein content (Bradford, 1976). The each remainder supernant was used in triplicate for AChE activity quantification by spectrophotometric analysis following the reaction of thiocholine (substrate) with DTNB on the increase of 5-mercapto-2-nitrobenzoate (yellow color) formation at 405 nm ($\varepsilon = 13.6\ mM^{-1}.cm^{-1}$) (Ellman *et al.*, 1961) and is expressed as $nmol.mg^{-1}$ total $protein.min^{-1}$.

6.2.6 ALP Analysis

Sex-differentiated gonads (n = 10) were homogenized separately on ice with 25 mM Hepes–NaOH buffer (pH 7.4) (125 mM NaCl + 1 mM DTT + 1 mM EDTA) and centrifuged for 30 minutes (2ºC) at 12,000 × *g*, resulting pellets were discarded. An aliquot of each supernant was collected to determine total protein content (Bradford, 1976) and another was adjusted with 35% acetone and later centrifuged for 5 minutes at 10,000 × *g*. Resulting pellets were dissolved with 1 M NaOH and subjected for 30 minutes to a 60ºC heating bath following an adaptation from Blaise *et al.* (1999). Phosphomolybdenum spectrophotometric method was applied in the measurement of inorganic phosphate (KH_2PO_4) concentration at 660 nm (Stanton, 1968) and ALP concentration expressed as µg $[PO_4].mg^{-1}$ total protein.

6.2.7 Statistical Analysis

The present results are expressed as mean ± standard deviation at each set of time. Two-way ANOVA was performed using SIGMAPLOT® to test differences between treatments (controls and each mixture exposure) within each and between tissues at each set of time. Ad-hoc Holm-Sidak was used on each biomarker differences discrimination with time, within and when applicable between gills and digestive gland tissues. Pearson correlations were performed using XLSTAT® 2012 to verify each biomarker dependency between controls and each mixture treatment and between mixtures. PCAs were performed using XLSTAT® 2012 to assess the variability associated to each biomarker factorial weight (SOD, CAT, GR, GST, LPO and CI) at each set of time and treatment group (control, mixture 1 and 2) between and within tissues. Finally, a PCA was also applied on the overall comparison of biomarker responses (SOD, CAT, GST, LPO and CI) to single IBU, DCF and FLX exposure with MIX 1 and 2. Statistical significance was defined at $p < 0.05$ level.

6.3 Results

6.3.1 Condition Index

No significant differences were found between controls and/or mixtures ($p > 0.05$) ranging between 14.2 ± 3.6 % to 15.8 ± 3.0 % in controls, 14.9 ± 3.7 % to 16.2 ± 2.5% in MIX 1 and 13.4 ± 2.5 % to 15.9 ± 6.1 % in MIX 2, respectively.

6.3.2 Antioxidant Enzymes

Generally the antioxidant response was more pronounced towards MIX 1 than MIX 2 in both tissues in which controls were mostly steady throughout time (Figure 6.1).

In exposed gills (Figure 6.1A), SOD activity decreased in both mixtures during the first week compared to controls although not significantly ($p > 0.05$). After two weeks exposure SOD activity in MIX 1-exposed gills increased significantly (2-fold higher than on the 3rd day) around 2-fold higher activities than controls and MIX 2-exposed gills respectively ($p < 0.05$). In exposed digestive gland (Figure 6.1B), no differences were found on the 3rd day in SOD activity ($p > 0.05$). This was followed by a 4-fold increase towards the first week in MIX 1-exposed digestive gland which in turn was 4

and 3-fold higher than controls and MIX 2- respectively ($p < 0.05$). By the end of the experiment, SOD activity decreased to control levels in MIX 1 exposure digestive gland (3-fold lower than previously observed) while in MIX 2 SOD increased slightly but not significantly from controls and MIX 1 exposure. When comparing the effect of mixture exposure between tissues gills controls' exhibited significantly higher SOD activity throughout time than digestive gland', while in opposition SOD activity was significantly higher in MIX 1-exposed mussels' digestive gland over the first week and in gills over the second week.

CAT activity in gills (Figure 6.1C) followed the SOD profile and was quite stable over the first week regarding both mixtures, showing a slight increment by the second week in MIX 1-exposed gills (2-fold higher than on the 3rd day) which even though significantly higher than MIX 2-exposure ones ($p < 0.05$) both were not different from controls ($p > 0.05$). In the digestive gland (Figure 6.1D) from the 3rd day to the first week, CAT activity increased 2-fold within MIX 1 and decreased 2-fold in MIX 2-exposed ones ($p < 0.05$). Over the 7th day, CAT activity in MIX 1-exposed digestive gland was significantly higher than in controls and MIX 2-exposed ones (2 and 4-fold respectively) whereas over the second week CAT activity decreased 2-fold in MIX 1-exposed digestive gland reaching controls and MIX 2-exposure levels. Finally, CAT activity was quite similar between tissues except for the significant enhancement in MIX 1-exposed digestive gland over the first week.

Alike for SOD and CAT, in gills GR activity (Figure 6.1E) showed a similar evolution pattern, in which over the course of the first week no significant differences were observed between controls and mixtures, followed by a 3-fold increase in MIX 1-exposed gills being at the same time 2-fold higher than controls and MIX 2-exposed gills ($p < 0.05$). In digestive gland (Figure 6.1F), GR activity varied significantly within controls, nonetheless after a slight inhibition tendency on the 3rd day shown in both exposure mixtures ($p > 0.05$) GR activity increased significantly within both mixtures-exposed digestive gland by the first week (approximately 3-fold) being also significant between them (higher after MIX 2) exposure. This was followed by a

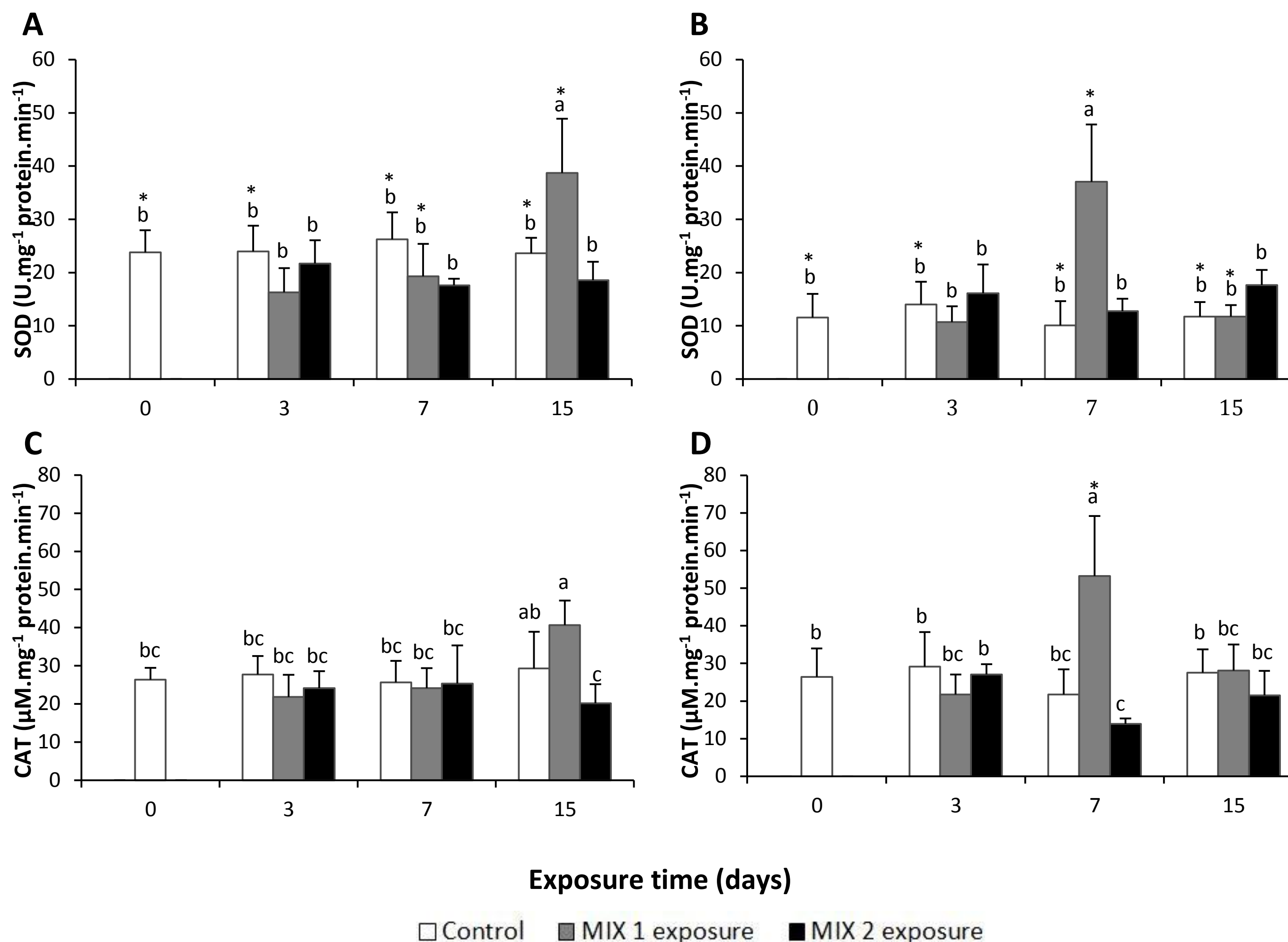

Figure 6.1: Antioxidant enzymes activities (mean ± standard deviation) in controls, mixtures 1 and 2 exposure of *M. galloprovincialis* tissues. SOD activity in gills (A) and digestive gland (B); CAT activity in gills (C) and digestive gland (D). Different letters express significant differences and bars signaled by an * express significant differences between females and males on respective day ($p < 0.05$). Note: Figure continues in the next page.

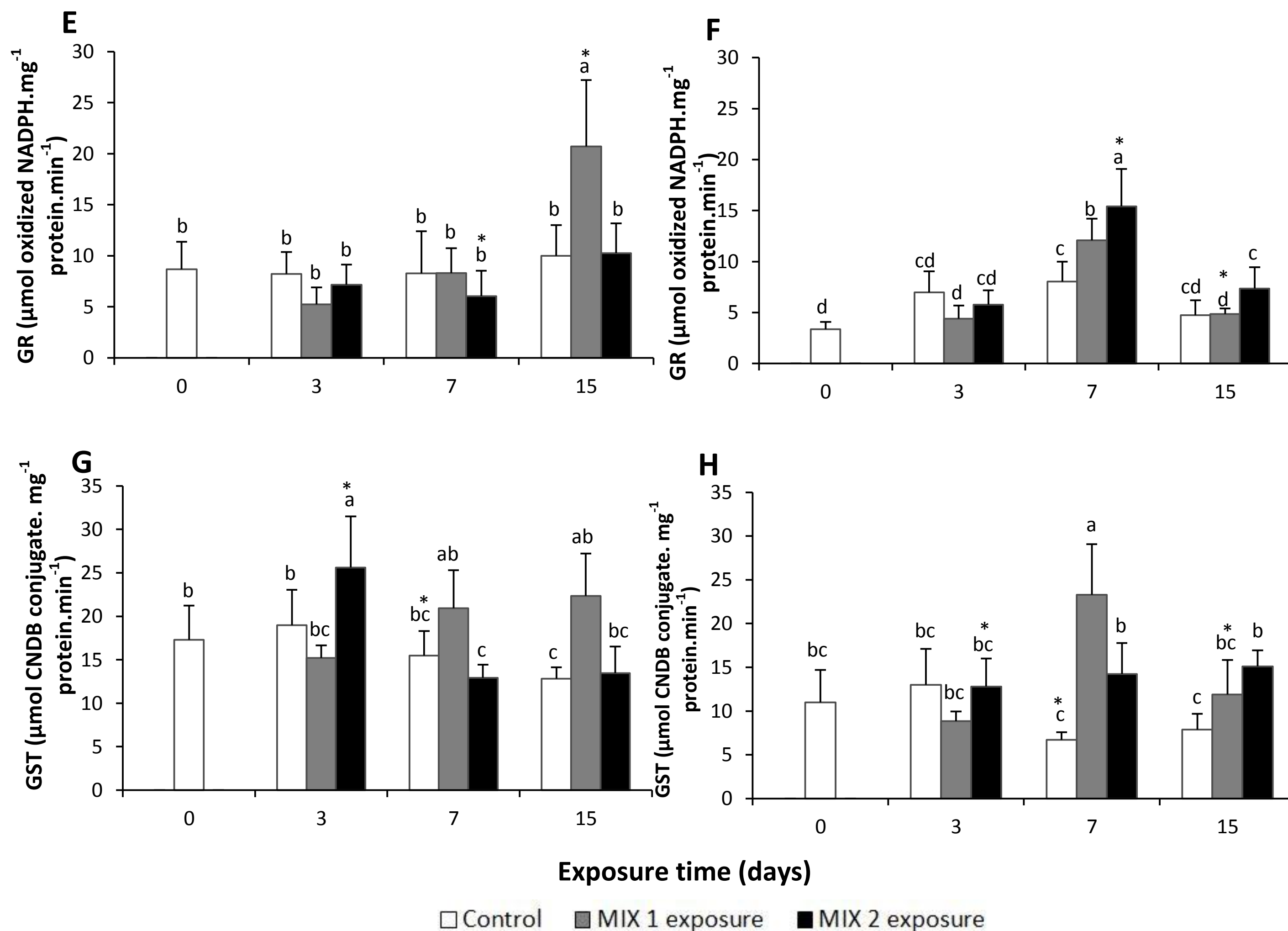

Figure 6.1 (Continuation): Antioxidant enzymes activities (mean ± standard deviation) in controls, mixtures 1 and 2 exposure of *M. galloprovincialis* tissues. GR activity in gills (E) and digestive gland (F); GST activity in gills (G) and digestive gland (H). Different letters express significant differences and bars signaled by an * express significant differences between females and males on respective day ($p < 0.05$).

significant 2-fold decrease of GR within each mixture, reaching controls activity by the end of the experiment. Regarding differences between tissues, in MIX 2-exposed gills GR activity was significantly lower than in digestive gland after one week, while over the second week MIX 1-exposed digestive gland exhibited significantly lower GR activity than MIX 1 exposed gills.

In gills Phase II GST activity (Figure 6.1G) decreased significantly in controls over the second week compared to the first days. Nevertheless a different behavior was observed between mixtures exposed gills, in which from the 3rd day to the first week MIX 1 GST activity enhanced over time ($p > 0.05$) while a significant 2-fold decrease occurred in MIX 2-exposed gills from the 3rd to the remaining days ($p < 0.05$). Even though in opposing manner GST activity in exposed gills was 2-fold different between mixtures over the 3rd and 7th day ($p < 0.05$). Moreover, after two weeks only for MIX 1-exposed gills GST activity was 2-fold higher than controls ($p < 0.05$). In digestive gland (Figure 6.1H) controls also decreased over time ($p < 0.05$). In this case, GST activity was steady in MIX 2-exposed digestive gland though higher than controls after the first week ($p < 0.05$). Whereas for MIX 1-exposed digestive glands GST activity presented a 3-fold enhancement between the 3rd and 7th day which in turn was 4 and 2-fold higher than control and MIX 2-exposed digestive gland ($p < 0.05$), this was followed by a significant 2-fold decrease in GST activity after two weeks yet not different from controls.

When comparing GST activity between tissues, exposed gills revealed significantly higher activities than digestive gland when exposed to MIX 1 over the 3rd day and towards MIX 2 after two weeks. Additionally controls were not different between tissues except for the decreased GST activity on digestive gland control' after the first week. Finally, SOD activity is directly related with CAT and GR activities in gills ($r = 0.925$ and $r = 0.895$) and in the digestive glands SOD is directly related to both CAT and GST ($r = 0.860$ and 0.912) which in turn are directly related between them ($r = 0.680$).

6.3.3 LPO

In gills (Figure 6.2A) LPO levels were consistently higher towards MIX 2 comparing to controls ($p < 0.05$) and except for the 3rd day to MIX 1-exposed ones. In MIX 1-exposed gills LPO levels remained unchanged and higher than controls throughout the exposure time ($p > 0.05$) being only 2-fold significantly higher by the second week. Whereas in MIX 2-exposed gills LPO levels enhanced linearly (LPO (μmol MDA. g^{-1} protein) = 78.9 t (days) + 516.6, $r = 1$) being 2 and 3-fold higher than first and second week MIX 1-exposed gills and 2 to 7-fold higher than controls over time ($p < 0.05$). In the digestive gland (Figure 6.2B) LPO levels did not vary between controls and mixtures until the first week, showing an abrupt increase in MIX 2-exposed digestive glands (7-fold higher than control and MIX 1-exposed ones). Finally, when comparing LPO levels between tissues, exposed gills exhibited consistently higher LPO levels than digestive gland (except after the second week) being at the same time directly related between them ($r = 0.863$).

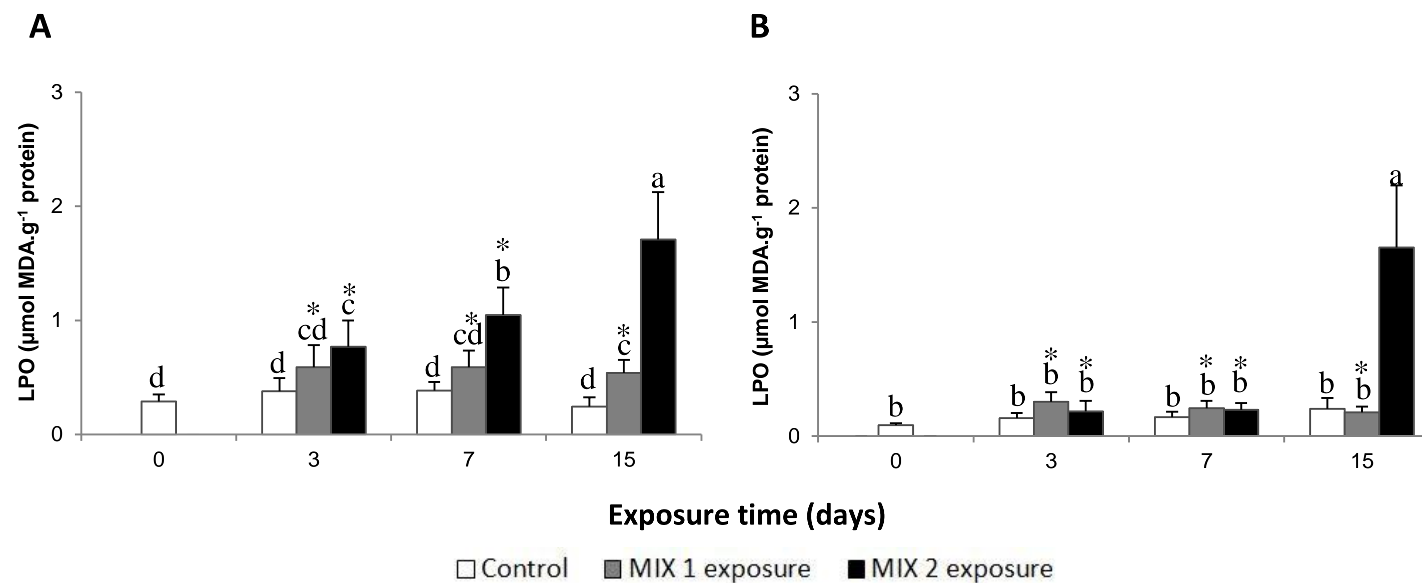

Figure 6.2: LPO (mean ± standard deviation) in mussel *M. galloprovincialis* controls, mixtures 1 and 2 exposure in gills (A) and digestive glands (B) over 15 days. Different letters are significantly different and bars signaled by an * express significant differences between tissues on respective day (p < 0.05).

6.3.4 AChE

No significant differences of AChE activity (Figure 6.3) exist between controls and mixtures. Nevertheless both mixtures exhibited an enhancement tendency throughout time, particularly MIX 2-exposed gills ($p > 0.05$). Additionally, a direct relationship exists between AChE activity, LPO levels in gills ($r = 0.806$) and digestive gland ($r = 0.789$).

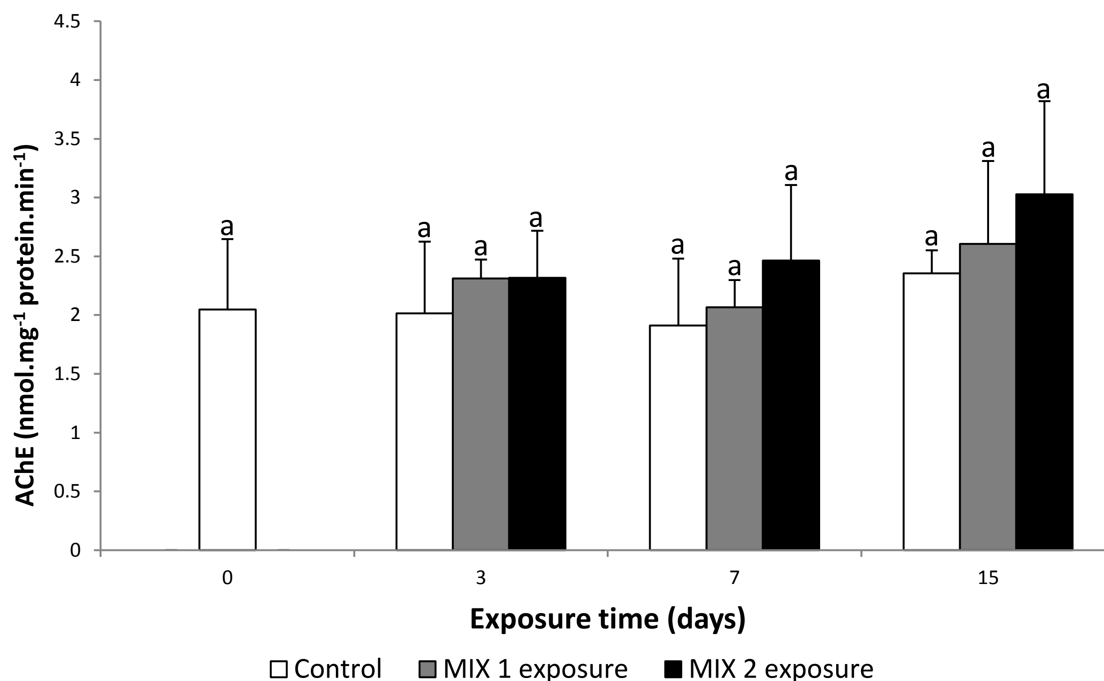

Figure 6.3: AChE activity (mean ± standard deviation) in controls, mixtures 1 and 2 exposed *M. galloprovincialis* gills during 15 days. Different letters express significant differences ($p < 0.05$).

6.3.5 ALP

ALP levels in female controls (Figure 6.4A) varied only after the second week being approximately 2-fold higher than remaining 3rd and 7th day ones, whereas male controls' (Figure 6.4B) exhibited a 2-fold lower level over the 3rd day than the ones after the following weeks ($p < 0.05$). Moreover the cross-comparison between genders only revealed a significantly lower ALP control level in males over the 3rd day.

Regarding exposed-females gonads while on the 3rd and 7th day MIX 1 showed 3-fold higher ALP levels than controls ($p < 0.05$), MIX 2-female gonads exhibited no differences over the 3rd neither from MIX 1 nor control which was followed by 3-fold higher level than control over the first week. Finally, after the second week no differences exist between controls and exposed female gonads ($p > 0.05$).

In males (Figure 6.4B), generally no significant differences exist found between mixtures and controls over the 3rd day. Over the first week MIX 2-exposed males exhibited 2-fold lower ALP levels than MIX 1-exposed ones though not different from controls. Additionally, ALP levels were enhanced throughout time in male gonads exposed to MIX 1 and unaltered in the ones exposed to MIX 2. The comparison between genders revealed 2-fold higher ALP levels in females than males when exposed to MIX 1 and MIX 2 respectively after the 3rd day and 7th day ($p < 0.05$) being not significantly different by the end of the experience. Finally, a direct relationship exists between AChE and ALP in females ($r = 0.695$).

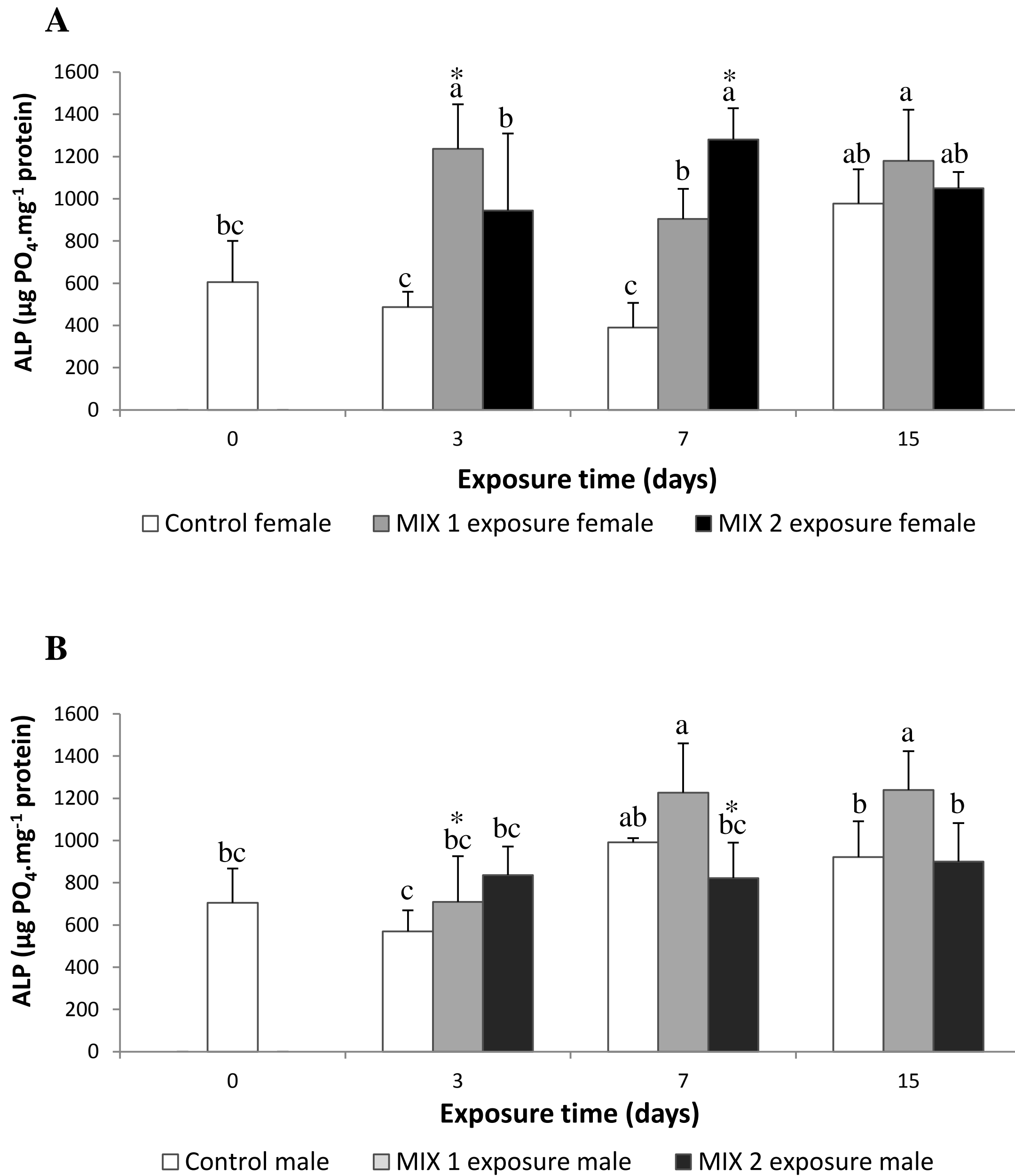

Figure 6.4: ALP levels (mean ± standard deviation) in controls, mixture 1 and mixture 2 exposed *M. galloprovincialis* sex-differentiated gonads (A – females, B – males) during 15 days. Different letters express significant differences and bars signaled by an * express significant differences between females and males on respective day ($p < 0.05$).

6.3.6 PCA in Gills and Digestive Gland

PCA (Figure 6.5A) expresses 72% of total variance (Tvar) in gills highlighting primarily 1) a gills controls cluster due to lower biomarker activities associated to GST activity unchanged from remaining treatment groups, 2) the dispersal of day 15 MIX 1-exposed gills was associated to the concomitant enhancement of the antioxidant enzymes SOD, CAT and GR activities, 3) day 3 and 7 in MIX 1-exposed gills unaltered biomarker responses and/or its inhibitory tendency when compared to controls and 4) MIX 2-exposed gills associated with the progressive enhancement of directly related LPO levels and AChE activity particularly over day 15. In digestive gland PCA (Figure 6.5B) represents 78% of Tvar and expresses 1) day 7 MIX 1- exposed digestive gland dispersal due to the concomitant enhancement of directly related SOD, GST and CAT activities, 2) controls cluster with remaining MIX 1-exposed digestive glands due to the overall unaltered biomarker responses and 3) day 7 MIX 2-exposed digestive gland associated to unaltered SOD, GST activities with a higher GR activity opposing a lower CAT activity and 4) day 3 and 15 MIX 2-exposed digestive gland due to higher CI and LPO levels. The overall PCA (Figure 6.5C) using gills and digestive gland together represents 68% of Tvar and highlights mostly 1) the dispersal of biomarker responses between tissues in which gills showed higher biomarker responses than digestive gland except for day 3 MIX 1- exposed gills and day 7 MIX 1- exposed digestive gland, 2) the overlapping dispersal of MIX 1-exposed day 7 digestive glands and day 15 gills due to the enhancement of directly related SOD, CAT and GR activities, 2) day 15 MIX 2-exposed tissues further influence by higher LPO levels opposing to an overall unchanged of biomarker responses, 3) day 3 MIX 2 and day 7 MIX 1-exposed gills due to higher GST activities and LPO levels enhancement concomitant with overall unaltered antioxidant responses, 4) gills controls cluster regarding lower SOD, GR, CAT activities responses, 5) MIX 1 and 2-exposed digestive glands showed no alterations of biomarker responses along with the ones given by controls.

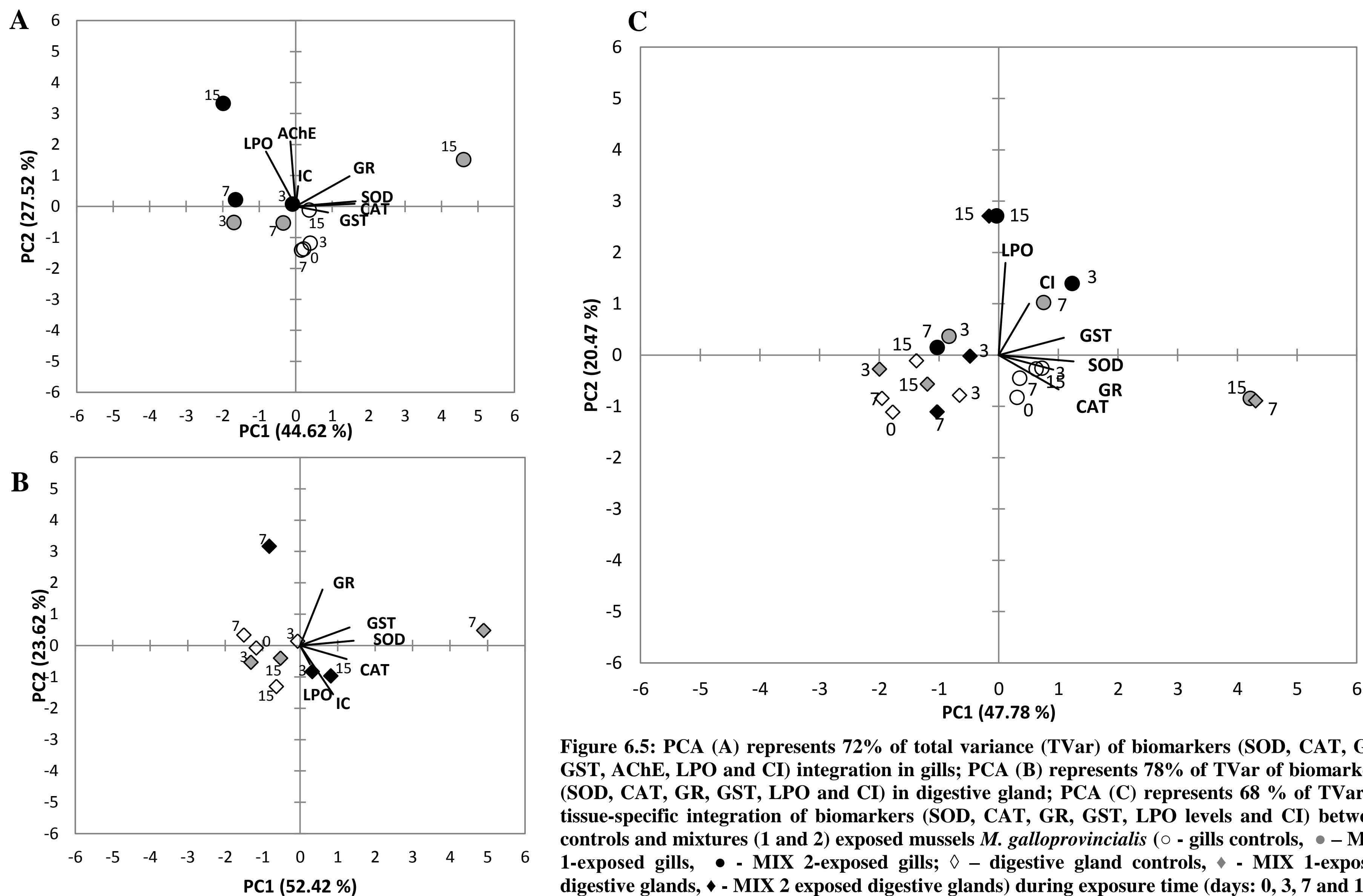

Figure 6.5: PCA (A) represents 72% of total variance (TVar) of biomarkers (SOD, CAT, GR, GST, AChE, LPO and CI) integration in gills; PCA (B) represents 78% of TVar of biomarkers (SOD, CAT, GR, GST, LPO and CI) in digestive gland; PCA (C) represents 68 % of TVar of tissue-specific integration of biomarkers (SOD, CAT, GR, GST, LPO levels and CI) between controls and mixtures (1 and 2) exposed mussels *M. galloprovincialis* (○ - gills controls, ● – MIX 1-exposed gills, ● - MIX 2-exposed gills; ◊ – digestive gland controls, ♦ - MIX 1-exposed digestive glands, ♦ - MIX 2 exposed digestive glands) during exposure time (days: 0, 3, 7 and 15).

6.4 Discussion

Mussels possess an open circulatory system continually exposed to the fluctuation of multiple stressor concentrations (Pipe *et al.*, 1999) which impact assessment data, particularly linking APIs, is extremely challenging and deficient. To our knowledge this is the first study addressing environmental realistic concentrations exposure to IBU, DCF and FLX (with and without Cu) mixtures effects in mussels *M. galloprovincialis*. The results showed that mixtures do not induce detriment on mussels' CI although promoting differential antioxidant enzymatic responses, damage and endocrine disruption effects.

Antioxidant enzymatic activities are universally applied as effective biomarkers of susceptibility to environmental contaminants due to their ability to counteract derived-ROS increase through the upkeep between pro- and antioxidant agents balance essential for cellular homeostasis (Livingstone, 2001; Lau *et al.*, 2004; Funes *et al.*, 2006; Valavanidis *et al.*, 2006).

In gills, only in MIX 1 exposure, SOD, CAT and GR activity were upregulated by the end of the exposure while an inhibitory tendency or unchanged of these enzymes occurred in those exposed to MIX 2 throughout the time (Figure 6.1A, C and E and 6.5A). Therefore Cu presence in MIX 2 led to an antagonistic effect by preventing, in particular, SOD activity conversion of superoxide anion to H_20_2 and consequently to the depletion of CAT activity substrate and GR inability to enhance antioxidant GSH as an additional free-radical scavenger or antioxidant enzyme co-factor (Figure 6.1) (Regoli and Principato, 1995; Regoli *et al.*, 2002; Geret *et al.*, 2002; Maria and Bebianno, 2011). Moreover, although Phase II GST activity was swiftly enhanced over the first days in MIX 2-exposed gills and only by the end of the experiment in MIX 1-exposed ones (Figure 6.1G), the conjugation reaction to convert these multi stressors into more excretable conjugates with free-GSH in the presence of Cu was also insufficient to prevent stronger damage evidences of LPO in this tissue (Figure 6.2A) (Regoli and Principato, 1995; Regoli *et al.*, 2002; Manduzio *et al.*, 2004). Contrarily with what was expected, in digestive gland the inducibility of antioxidant enzymes was swifter that in gills [since this organ is the first in contact with the contaminants presented in the water] (Figure 6.1). This is owed however, to the fact that most antioxidant enzymes appear to be inhibited in gills over the first week, triggering these enzymes to act

initially in the digestive gland on the detoxification of ROS. Either showing higher induction and direct relationship of SOD, CAT and GST activities after 7 days of exposure to MIX 1 (Figure 6.1B, D and H) or by higher inducibility of GR activity and CAT activity inhibition on exposed digestive gland after MIX 2 exposure (Figure 6.1B and F). Furthermore GST activity in MIX 2-exposed digestive gland was consistently enhanced after the first week (Figure 6.1 H) emphasizing Phase II conjugation reaction with GSH to enhance formed metabolites elimination. Though, the joint action of antioxidant enzymes in digestive gland prevented LPO during the exposure to both mixtures over the first week, over the following week the antioxidant system defense in digestive gland exposed to MIX 2 failed to counteract ROS derived from Cu presence through an abrupt enhancement of LPO in this tissue.

In summary, compared to digestive gland, gills tissue revealed to be more affected by mixtures exposure showing higher damage throughout the time owing to either delayed antioxidant system responses after the exposure to MIX 1 or by its enzymatic activities inactivation after MIX 2. Nonetheless, the prolonged exposure to the latter showed Cu additional impact blocking the antioxidant system ability to prevent LPO induction. This in agreement to the fact that this metal is known to produce LPO in mussel species by converting harmless oxidants into toxic ROS through Fenton-type reactions (Figure 1.9) (Viarengo *et al.*, 1990; Lau *et al.*, 2004; Maria and Bebianno, 2011).

As mentioned before, ALP levels alteration are an endocrine disruption biomarker (Blaise *et al.*, 1999; Porte *et al.*, 2006; Ortiz-Zarragoitia and Cajaraville, 2006; Matozzo and Marin, 2008; Matozzo *et al.*, 2008). ALP levels were enhanced by both mixtures in females (Figure 6.4A); at day 3 firstly onset by MIX 1, then over the first week inverting to a higher induction by MIX-2 pointing out to the increase of Vtg-like proteins synthesis in exposed gonads. In males (Figure 6.4B), the effect of mixtures is not so evident although MIX 1 yield to higher and continuously elevated ALP levels than MIX 2. Though control values variation with time rendered to unclear or masked results by the end of the exposure period, both mixtures clearly induced time-specific vitellogenin-like proteins in both sex-differentiated gonads, highlighting an effective endocrine disruptor effect of these contaminants at environmental realistic concentrations.

When comparing antioxidant system responses, mixtures of APIs exerted different tissue-specific enzymatic activities than single exposures (Chapters 3-5) further confirmed in Figures 6.6A and B[8].

In gills (Figure 6.6A), the integration of all data, reveals different time and API specific dispersal distribution in which for single exposure IBU the most distinct integrated effect happened after the first week of exposure related to higher SOD activity and LPO levels. The same could be stated (although to a lesser extent) for DCF single exposure (after 3 days) and MIX 2 (after two weeks) most related to LPO levels increase. Whereas, for FLX and MIX 1 exposures the integration revealed higher relevance of GST and CAT activities increase either after the first and second week respectively.

In digestive gland (Figure 6.6B), the most distinct effects are related to higher LPO levels after both FLX and MIX 2 exposures after two weeks and to a much lesser extent after the first 3 days of DCF exposure, higher CAT activity for IBU single exposure clustering between the 3rd and 7th day, higher SOD and GST activities after the first week of exposure to MIX 1 and DCF.

[8] Note: GR activity was not included since no data exists for single FLX exposed mussel' tissues

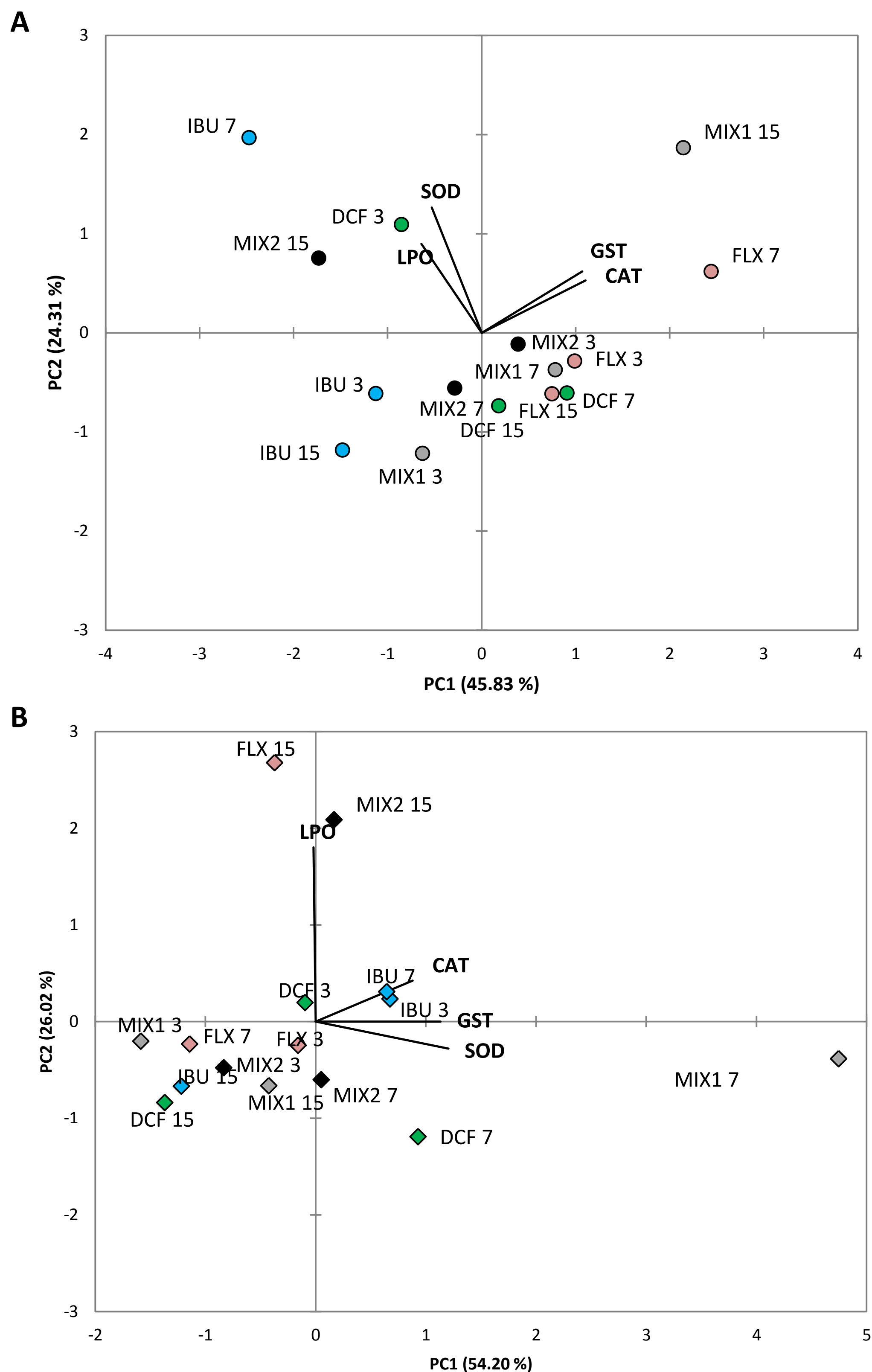

Figure 6.6: PCA (A and B) represents 70 and 80% of TVar of tissue-specific integration biomarkers (SOD, CAT, GST, LPO) in mixtures and single API exposed mussels *M. galloprovincialis* gills (A) and digestive gland (B) in relation to each time-control (● – IBU exposed gills, ● –DCF exposed gills ● – FLX exposed gills, ● – MIX 1-exposed gills, ● - MIX 2-exposed gills; ◊ – digestive gland controls, ♦ - IBU-exposed digestive glands, ♦ - DCF-exposed digestive glands, ♦ - FLX-exposed digestive glands ♦ - MIX 1 exposed digestive gland, ♦ - MIX 2 exposed digestive glands) during exposure time (0, 3, 7 and 15 days).

6.4.1 Mixture components interactions

Herein follow the proposed interactions between mixture components affecting each biomarker behavior.

6.4.1.1 SOD activity

SOD activity in MIX 1 exposed gills (Figure 6.1A and 6.6A) relates mostly to FLX activity down regulation (Figure 4.1A) which is antagonized by NSAIDs IBU and DCF up regulation (Figures 3.1A and 5.1A) resulting in an inhibitory tendency of this enzyme activity whereas, Cu in MIX 2 counteracts the effect of FLX and renders SOD activity to control levels. Over the next week, the noticeable increase of SOD after IBU single action is independent from both MIX 1 and 2 (inhibition tendency) antagonized by the synergism between the down regulation after DCF and FLX exposure and/or Cu as observed by Maria and Bebianno (2011) promoting, as at the beginning of the experiment, the inhibition tendency of SOD activity. By the end of the experiment, the enhancement of SOD in MIX 1-exposed gills is due to a potentiation of IBU independent action (up regulation tendency) antagonistic to FLX and DCF downregulation effects, while the presence of Cu cancels IBU independent action, rendering SOD to an activity slightly lower than controls.

In MIX 1-exposed digestive glands (Figure 6.1B and 6.6B), over the first 3 days, the NSAIDs SOD activity increase is antagonized by FLX single exposure SOD down regulation behavior which alike for exposed gills leads to a inhibitory tendency of SOD activity. Inversely, over the first week, the addition of NSAIDs single exposure SOD activity up regulation is potentiated, resulting on a significant increase of this enzyme activity in MIX 1-exposed digestive gland while FLX exposure down regulation ($p > 0.05$) has no effect in the mixture. Finally by the end of the experiment, the addition of each single APIs exposure independent action results in the significant decrease of SOD activity to basal levels after MIX 1; whereas in MIX 2-exposed digestive glands, the presence of Cu induces a slight, although not significant, increase of SOD activity compared to APIs mixture after the 3rd and 15th day. Furthermore, the enhancement of SOD activity due to Cu was only observed in digestive gland exposed to 10 µg Cu.L^{-1} single exposure over 15 days (Gomes *et al.*, 2012), while the exposure to the same concentration (5 µg.L^{-1}) over one week resulted in the inhibition of SOD in both tissues

(Maria and Bebianno, 2012). Therefore the presence of Cu in MIX 2 reveals different SOD activity behavior from single exposure essays confirming its antagonistic action in gills and synergistic action in digestive glands.

6.4.2 CAT activity

In mixtures-exposed gills (Figure 6.1C and 6.6A), over the first 3 days, the CAT activity inhibitory tendency results clearly from the addictive effect of the three APIs (Figure 3.4A, 4.1C and 5.1C). Over the next week the synergetic effect of NSAIDs CAT inhibition is counteracted by FLX induced CAT activity) either with (MIX 1) or without Cu (MIX 2) rendering CAT activity to basal levels. The enhancement of CAT activity was also observed after 5 $\mu g.L^{-1}$ Cu single exposure for 7 days (Maria and Bebianno, 2011). By the end of the experiment, the slight increment of CAT activity in MIX 1-exposed gills follows the FLX single exposure effect (inverse to previous days) cancelling the synergetic down regulation effect of NSAIDs. The opposite was observed after MIX 2 exposure with a significant CAT activity down regulation owed to Cu and NSAIDs inhibitory synergism counteracting FLX single exposure up regulation.

In mixtures exposed-digestive gland (Figure 6.1D and 6.6B), over the 3rd day an antagonistic behavior between all three single APIs exposure promotes CAT activity up regulation (Figure 3.4B, 4.1D, 5.1D) resulting in slight inhibitory tendency of this enzyme activity in both mixtures, in the MIX 2 case, Cu presence induced a slight increment of SOD rendering in a less pronounced CAT downregulation than MIX 1. While over the next week, the increase of CAT activity in MIX 1-exposed digestive gland results from the antagonism between DCF down regulation tendency (Figure 5.1D) and the synergism between FLX and IBU CAT activity up regulation (Figure 3.4B and 4.1D). On other hand in MIX 2-exposed digestive gland CAT activity decrease results from the antagonistic effect of IBU and FLX upregulation against the potentiation of DCF downregulation by Cu. By the end of the exposure time, after MIX 2 exposure, digestive glands show an addictive behavior between mixture components translated in a slight CAT decrease, while in MIX 1 exposed ones, the unchanged of CAT activity results from the antagonism between NSAIDs and FLX.

6.4.3 GR activity

Since GR activity FLX single effect data is missing, it is very difficult to ascertain mixtures components behavior. Nevertheless, GR activity up regulation in MIX 1-exposed gills (Figure 6.1E and 6.6A) by the end of the experiment was more similar with the one after the 3rd day of DCF single exposure (2-fold higher in relation to basal levels) (Figure 5.1E) and antagonistic with IBU single exposure down regulation (Figure 3.1C) over time. In MIX 2-exposed gills, GR activity remained mostly unchanged throughout the experiment probably due to antagonistic effects between IBU exposure (GR activity downregulation) and remaining mixture components particularly due to Cu single exposure since it induced the up regulation of GR activity in this tissues (Maria and Bebianno, 2011).

GR activity in both mixtures-exposed digestive gland in the first 3 days (Figure 6.1F and 6.6B) seem to follow IBU single exposure down regulation independent action (Figure 3.4C) which is antagonistic with that of DCF exposure (up regulation) ($p < 0.05$) (Figure 5.1F) while Cu probably cancelled IBU downregulation effect. Over the first week, mixtures followed instead DCF single exposure up regulation effect antagonist to IBU single exposure. In the case of MIX 2, the increase of GR activity was stronger due to the presence of Cu however inverse with that observed in single Cu exposed-digestive glands, which showed the down regulation of GR activity after one week (Maria and Bebianno, 2011). By the end of the experiment, NSAIDs single exposure show an antagonistic effect rendering GR activity to basal levels in MIX 1-exposed digestive glands, while the presence of Cu induced its slight enhancement over APIs antagonistic effects.

6.4.4 GST activity

In MIX 1-exposed gills, over the 3rd day GST activity (Figure 6.1G and 6.6B) follows the addictive effect between APIs components (Figure 3.1D, 4.1E, 5.1G) resulting in an slightly decrease of GST activity, whereas at the same time MIX 2-exposed gills GST activity is enhanced probably due to Cu synergism with both FLX and DCF single exposure (up regulation effect) antagonizing IBU downregulation effect. After one week, the GST activity up regulation tendency in MIX 1-exposed gills results from the antagonism between up regulation additive effect of FLX and DCF single exposures with single IBU exposure (GST activity down regulation) . Inversely in MIX 2-gills, the

addictive effect of APIs with Cu resulted in GST activity down regulation tendency. By the end of the exposure period, the GST up regulation activity in MIX 1-exposed gills results from the potentiation of DCF single exposure up regulation (unaltered neither by single IBU nor FLX exposure down regulation), whereas the presence of Cu in MIX 2 blocks this interaction rendering GST activity recovery to basal levels.

In mixtures-exposed digestive gland (Figure 6.1H and 6.6B), over the first 3 days the up regulation of single NSAIDs and FLX exposures (Figure 3.4D, 4.1H and 4.1F) did not change significantly GST activity, pointing out to antagonistic effects between mixture components. In the first week, both mixtures show enhanced GST activity due to a probable potentiation of NSAIDs exposures up regulation which was not significantly affected by FLX single exposure down regulation tendency. Nevertheless at this time, the effect of Cu in MIX 2 resulted in a less pronounced increment of GST activity. By the end of the exposure, MIX 1-exposed digestive gland showed GST activity up regulation tendency. This is due to the up regulation synergism between FLX and DCF which was not counteracted by IBU independent down regulation action. The same happens after MIX 2 exposure, where the significant GST activity up regulation is due to the addictive effect of Cu with FLX and DCF.

6.4.5 LPO level

LPO levels in both mixtures-exposed gills (Figure 6.2A and 6.6A) follow the addictive effect of single API exposure (Figure 3.2, 4.2A, 5.2A) resulting in the enhancement of LPO levels though only significant after MIX 2 exposure. Over the first week, MIX 1 LPO levels are slightly enhanced due to a counter effect between IBU and DCF single exposures (Figure 3.2 and 5.2A) and similar FLX independent action, whereas in MIX 2 LPO level is increased by IBU and FLX synergism with Cu, as reported by single Cu exposure LPO increase effect (Maria and Bebianno, 2011). By the end of the exposure period, as observed for the first 3 days, both mixtures induce a significant LPO levels increase resulting from the addictive effect of APIs, this is most evident after MIX 2 by Cu ability to potentiate LPO levels.

In mixture exposed-digestive glands (Figure 6.2B), LPO levels remain either slightly enhanced (MIX 1) or unchanged (MIX 2) not following the addictive effect of single APIs exposure LPO upregulation (Figure 3.5, 4.2B, 5.2B) i.e. neither IBU nor DCF LPO increase independent action had effect after MIX 1 and no addictive effect was

associated to a probable Cu LPO level increase after MIX 2 exposure. In the first week, LPO levels remain unchanged in both mixtures, nevertheless showing evidence of an antagonism between IBU and DCF single exposures (up and down regulation effects respectively) following instead, as observed in gills, a behavior similar to FLX single exposure independent action. Finally, by the end of the experiment while in MIX 1-exposed digestive glands LPO levels remain unaltered due to the antagonistic effect between the three APIs, in MIX 2 exposure ones, Cu is antagonistic to IBU rendering LPO levels similar to the addictive effect between FLX and DCF LPO levels.

6.4.6 AChE activity

Although no significant alterations were found in AChE activity, both mixtures showed a progressive AChE enhancement. In fact, FLX and DCF single exposures[9] seem to lead to an AChE activity induction particularly over the first 3 days (Figure 4.3 and 5.3). This highlights an alternative evidence of nerve impulse transmission interference diverging from the general reported AChE activity inhibition after mussels exposure to organochloride pesticides, PAHs (like, benzo[a]pyrene), detergents, other APIs such as propranolol and paracetamol and several metals such as Cu (both as nanoparticles or ionic form) (Najimi *et al.*, 1997; Banni *et al.*, 2010; Solé *et al.*, 2010; Gomes *et al.*, 2011).

Several possible explanations were given in Chapters 4 and 5 concerning single exposure to FLX and DCF namely: the evidence of AChE activity increase during cell apoptosis (Zhang *et al.*, 2002); and particularly in the case of FLX single exposure both to a possible competition for ACh activation receptor after the increase of 5-HT concentration in cells and also an inverse relationship between AChE and estradiol levels (Tsuchiya *et al.*, 2004) since ALP levels were severely downregulated (Figure 4.4). Therefore, these results point out to an antagonistic effect between mixture components that prevented its enhancement in mussels as previously mentioned. Furthermore, in the case of MIX 2-exposed ones the evidence of single Cu exposure down regulation effect in these species due to its affinity to bind with AChE thiol residues (Najimi *et al.*, 1997; Gomes *et al.*, 2011) were particularly antagonized by APIs since AChE activity is slightly higher than in MIX 1 exposure ($p > 0.05$).

[9] no available data for IBU

6.4.7 Vitellogenin-like proteins (ALP levels)

In MIX 1-exposed females (Figure 6.4A) the enhancement of ALP levels in the first 3 days is owed to the potentiation of IBU and DCF single exposure up regulations (Table 3.4[10] and Figure 5.4) antagonizing FLX single exposure down regulation effect (Figure 4.4). The same effect happens although to a lesser extent after MIX 2 exposure. After the first week, ALP levels are derived from the synergetic effect between NSAIDs which continue to antagonize FLX single exposure down regulation effect. In the case of MIX 2-exposed females, Cu presence potentiates the synergism between NSAIDs enhancing even more vitellogenesis. By the end of exposure period, the up regulation of ALP observed after single DCF exposure is antagonized by the addictive effect of single FLX (down regulation) and IBU (up regulation) leading to the decrease of ALP to basal levels in both mixtures, particularly evident after MIX 2.

In MIX 1-exposed males, ALP levels enhancement results from an antagonistic effect of DCF (up regulation) and FLX (down regulation) with IBU single exposure which diminish IBU ability to increment ALP levels over the 3rd day of exposure. The presence of Cu, on other hand, potentiates the induction of ALP compared to MIX 1. Over the following week in MIX 1-exposed males ALP level is slight enhanced resulting from IBU up regulation effect antagonism with FLX and DCF down regulation effect, while after MIX 2 exposure an antagonism effect between the addictive effects of APIs in the presence of Cu is observed rendering ALP to slightly lower levels than controls. By the end of the experiment the addictive effect of APIs single exposure was somewhat potentiated in MIX 1-exposed males, while Cu presence antagonized this effect decreasing ALP levels to basal ones.

Finally, the ALP levels derived from mixtures are only significant in females and mostly following the interactions between NSAIDS, nevertheless Cu presence also enhanced vitellogenesis after the first week in exposed-females. Therefore, both mixtures have the potential to cause endocrine disruption in mussels.

[10] Note: clearly lower basal levels exist in IBU single exposure than the other single exposure assays between exposed individuals and respective controls.

6.5 Conclusions

APIs occur in the aquatic environment always as part of complex mixtures (see Chapter 1 and 2). This study revealed that multiple APIs stressors induce distinct effects in *M. galloprovincialis* depending on the nature of the mixture. While MIX 2 led to an overall increment of LPO levels (especially in gills) along with lower ability to induce antioxidant defense system and/or producing the inactivation of some antioxidant enzymes, MIX 1 exerted, in overall, higher antioxidant enzyme responses. Additionally, while neither MIX 1 nor 2 induced AChE activity alterations, both mixtures induced vitellogenin in females during the first week. Finally, even though, it was extremely hard to assess each individual component true contribution, several time and tissue-specific interactions were proposed for the overall biomarker alteration outcome.

6.6 References

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Chapter 7

General Discussion

7 General Discussion

The application of APIs in medical practice is undoubtedly one of the greatest achievements for life quality improvement in modern societies (Roberts and Bersuder, 2006). In Europe, more than 3000 types of APIs from of all therapeutic classes (e.g. analgesics, antiasthmatics, antibiotics, anticonvulsants/antiepileptics, antidepressants, antidiabetics, antihypertensive, anti-inflammatory drugs, antineoplastics, antipsychotics, antitumor agents, anti-ulcerants, beta-blockers, blood lipid lowering agents, lipid regulators, steroidal hormones, etc.) are daily applied in human and veterinary practice (Ternes *et al.*, 2004; Fent *et al.*, 2006). Novel and more efficient APIs are constantly being developed, and consumers demand has been growing in particular on the new emerging countries (such as China, Brazil and India) (IMS Health, 2010). Nevertheless worldwide, the use of APIs is at halfway of the desirable as more than 50% of the medicines are prescribed and taken incorrectly by the patients and half of the countries do not have suitable responsible rational use policies or if so, fail on its implementation (WHO, 2010; 2011).

The problematic around APIs in the environment arises mostly from the fact that after therapeutic application, these compounds are excreted almost in their native form or as metabolites, entering by diffuse (through aquaculture or agricultural run-off) or point (through hospital and domestic untreated and/or WWTPs effluents) sources into the aquatic systems (Heberer, 2002; Kümmerer, 2004; Bound and Voulvoulis, 2007; Kümmerer, 2009). Until recently, the lack of proper instrumental techniques (able to infer APIs presence in the environment) resulted on the overlook of its fate, occurrence and impact over the ecosystems and therefore on their classification as emergent environmental contaminants (Fent *et al.,* 2006). Subsequently, most WWTPs are ill-prepared for APIs removal or even consider its contamination in their treatment processes aggravating their ubiquitous cycle in the aquatic environment. Although several methods (such as increasing sludge age, ultrafiltration and ozonization) could be adopted fairly easily to improve APIs removal from WWTP effluents also measures that involve the hospital wastewater and urine treatment separation should be considered to greatly diminish APIs load in WWTPs (Ternes *et al.*, 2004).

Presently, almost two hundred APIs (from all therapeutic classes) have been screened worldwide in surface and ground waters as well as in public tap water at concentrations from ng.L^{-1} to µg.L^{-1} (Fent *et al.*, 2006; Kümmerer, 2009; Daughton, 2010; Santos *et al.*, 2010). Yet, as mentioned and stressed out in Chapter 2, since APIs assessment by conventional water screening methods continues to be severely limited, the application of passive integrative samplers (like POCIS) is increasingly regarded as a suitable alternative to better respond to Water Framework Directive (WFD – 2000/60/EC) criteria and demands (Togola and Budzinski, 2007; Dévier *et al.*, 2011). Most importantly, in an ecotoxicological perspective, APIs effects (particularly the chronic ones) assessment on non-target species is still rather unknown and far from readily predicted, since beside presenting multiple chemical structures, properties and modes of action, these contaminants are bioactive and occur as part of complex mixtures (Togola and Budzinski, 2007; Quinn *et al.,* 2008; Kümmerer, 2009; Kasprzyk-Hordern, 2010; Santos *et al.*, 2010). Accordingly, the main focus of this thesis, beside APIs quantification in Portuguese riverine water (Chapter 2), was to assess and provide new data concerning oxidative stress, neurotoxic and endocrine disruption effects applying a multibiomarker approach after the exposure of sentinel species *Mytilus galloprovincialis* to single and mixtures of three highly consumed APIs, NSAIDs ibuprofen and diclofenac and SSRI fluoxetine and the interaction with a traditional contaminant (Cu) at environmentally relevant concentrations (Chapters 3-6).

7.1 Occurrence of APIs in the Aquatic System: Arade River (Portugal)

In 2008, when this thesis first started, there was no information whatsoever concerning APIs in Portuguese surface waters, beside fluoroquinolone antibiotic in Mondego river (Coimbra, Portugal) (Pena *et al.*, 2007). Meanwhile, only two articles were published targeting and validating the quantification of 7 APIs (of which only FLX, CAR, DIA were concurrent with this study) in Douro riverine water (Oporto, Portugal) using SPE-LC–MS/MS method with ion trap detection (Madureira *et al*. 2009; 2010) and a very recent study focused only on NSAIDs IBU concentrations assessment in 12 rivers and Ria de Aveiro estuary located in the North of Portugal (max. concentration of 723 ng.L^{-1} in Lima river) (Paíga *et al.*, 2012). Since the analytical techniques commonly employed in this type of screening are extremely expensive and time-consuming it partially justifies the lack of information. Accordingly, it urged a further assessment of a

greater number of APIs from several different therapeutic classes in Portuguese waters. This motivated the intent to perform APIs quantification and presence profile in Arade river (one of the two major rivers in the South of Portugal) favoring the holistic TWA concentration assessment given by passive device samplers for polar compounds POCIS. Since there were no records to compare with, and POCIS handling and analysis methodology was still fairly recent (U.S. Patent #6,478,961 - by Petty *et al.*, 2002) it was necessary to perform a first screening (on 2009) at 2 different sites in Arade river (Section 2.2.1, Figure 2.3) for POCIS' preliminary validation using reference Rt pre-calibrated APIs (described in Table 2.2 and 2.3 and section 2.3.3). After this one-month validation study, a more comprehensive study followed at the same sites accessing the impact of tourism across the summer months (from August to November in 2010) on APIs concentration variation due to the expected overload of WWTP discharges.

First of all, analgesic ASP and antiasthmatic TER were not reconsidered in 2010 due to low recovery rates therefore the reference APIs list was reduced to 19 APIs (Table 2.2), from these neither antidepressants DOX and IMI, antiasthmatics CLE and SAL or NSAID KET were ever detected in Arade river. Moreover, antidepressant AMI was only identified in 2009 and anxiolytic BRO in 2010.

The comparison between years revealed that the overall average of APIs concentrations increased remarkably from 2009 to 2010, particularly for stimulant CAF (~10-fold higher) and antiasthmatic THEO (~ 5-fold higher) (with the exception of BRO which concentration was below the limit of detection) (Figures 2.6, 2.7 and 2.9 - 2.11). Likewise, it was noticed that CAF and THEO concentrations were the highest in Arade river (~ 1 $\mu g.L^{-1}$ and 200 $ng.L^{-1}$ respectively), followed either by analgesic PAR (~ 20 to 90 $ng.L^{-1}$) or NSAID IBU (between ~ 5 to 30 $ng.L^{-1}$) (Figure 2.6, 2.9 and 2.10). Also, while in 2009, anti-lipidemic GEM was alternatively the 4 or 5th highest (~ 2.5 to 5 $ng.L^{-1}$) detected API depending on the site (Figure 2.6), in 2010 anticonvulsant CAR (~ 10 to 30 $ng.L^{-1}$) presence was generally more preponderant (less than 1 $ng.L^{-1}$ in 2009) in both sites (Figure 2.10). NSAIDs NAP and DCF relative concentrations were similar within each year, though much higher in 2010 (from an average of less than 1 $ng.L^{-1}$ to one over 10 $ng.L^{-1}$) (Figures 2.7 and 2.10). Lastly, antidepressants and anxiolytics (BRO, NOR, FLX, AMI, DIA and ALP) were always detected at very low concentrations either below 1 or 3 $ng.L^{-1}$ in 2009 and 2010 respectively (Figure 2.7 and

2.11). Although it was difficult to ascertain whether abiotic parameters and the evident alteration in the trends of medical products consumption between years (Table 1.6) may be influencing these concentrations differences, it was assumed that the main contributing factor was the alteration performed in the extraction methodology by using RapidVap vacuum evaporation system to concentrate the samples (Section 2.3.2).

In terms of the impact of summer (Section 2.5.1), the results revealed to be quite inconclusive, with noticeable site and time-dependent individual APIs concentration differences. Some similarities between sites were noticeable though, namely a higher prevalence of psychoactive drugs in August (such as DIA), higher NSAID DCF in September and a concomitant highest presence of antiasthmatic THEO, analgesic PAR and anxiolytic ALP in October. All the other APIs concentrations were quite transient. It was highlighted that while at site 1 APIs monthly distribution was more heterogeneous (Figure 2.13), APIs concentrations were in overall higher at site 2 (with the exception of NSAID DCF and psychoactive drugs FLX and DIA). These differences between sites were explained as being due to the combination of a noticeable confinement of site 2 associated with a closer proximity to WWTP2 discharge and a higher dilution factor and hydrodynamics at the vicinity of site 1 which is at much greater distance of WWTP1 (Figure 2.2). Therefore the possible impact of summer was related to the continuous presence of APIs during the dry-season and especially by the highest concentrations of several APIs by the end of October which pointed out to uncontrolled discharges and possible overload of the two WWTPs since as suggested by Vystavna *et al.* (2012) the water run-off volume is expected to be low at that time of year. More so, due to the presence of high concentrations of CAF, which non-conservative behavior, usually renders high WWTP removal rates (Togola and Budzinski, 2007; Vystavna *et al.*, 2012).

Finally, the results of APIs' TWA concentrations were compared to similar works performed using POCIS or spot analysis in surface waters (Section 2.5.2 and 2.5.3 respectively). Several APIs' TWA concentration were found to be in agreement (Table 2.4) with Jalle river (France) and lower than in Lopan river (Ukraine) namely FLX, AMI and CAR, while others such as CAF, PAR, IBU and GEM were greatly higher in Arade river than the ones found in those rivers. Interestingly and inversely to that observed in the Portuguese river, NSAID KET was expressively reported in both rivers

while the highest detected antiasthmatic THEO in Arade river and anxiolytics DIA and ALP were below detection limit (Vystavna *et al.*, 2012). The concentrations of CAF, CAR and GEM in Arade river were also much higher than the ones reported by Munaron *et al.* (2012) in a coastal lagoon and semi-enclosed bay in French Mediterranean coast, which also identify the presence of KET and much lower concentrations of DIA and DCF. As the semi-enclosed bay is more confined and therefore subjected to longer water renewal times and more vulnerable to effluents discharge can explain the overall higher APIs TWA concentrations. Therefore, a similar situation might explain the differences in APIs concentration between Arade river' sites. Additionally, Arade river presented much lower concentrations of DCF, CAR than Ouse river (United Kingdom) and FLX in Crabtree Creek (USA) (Zhang *et al.*, 2008; Bringolf *et al.*, 2010).

Relatively to studies involving spot sampling (Table 2.5), concentrations of CAR and DIA and higher FLX concentrations in the Arade river were lower than that of Douro river (Madureira *et al.*, 2009; 2010). Although it was suggested that the difference between concentrations was due to different sampling techniques, it was also noticed that Arade river is in the vicinity of a much lesser densely populated area (8-fold lower than Douro river metropolitan area) (INE, 2011) showing nevertheless higher concentrations of the antidepressant. The same could be said in relation to Arade river' CAF and PAR concentrations being in agreement with Halifax watershed and Ebro river basin (Comeu *et al.*, 2008; Gros *et al.*, 2010) or as for several other Arade river APIs' concentrations that were in agreement with other areas worldwide. Therefore it was argued that even though APIs presence in water is dependent on numerous social-economic factors and cultural health practices that the ubiquity of these emergent compounds could be greatly diminished with the improvement of current WWTP works. Moreover, the results showed that POCIS clearly enabled the characterization of APIs concentrations in the Arade river (which was totally unknown), highlighting that the application of this passive sampler may become legible as the preferential sampling method to access these emergent compounds in the aquatic systems and should be further included in the WFD guidelines. Finally, as remarked by Madureira *et al.* (2010) even if the detected APIs concentrations are considered low to singularly cause acute effects this should not exclude the potential chronic effects caused by the continuous

exposure to APIs (either as single or mixture) on an economically and ecological relevant non-target species like mussels *Mytilus galloprovincialis*.

7.2 Effects of selected pharmaceuticals in mussel *M. galloprovincialis*

Present knowledge concerning APIs effects on non-target organisms is still severely limited, although considering the increasing interest given by the scientific community in the past decade (Kümmerer *et al.*, 2009; Santos *et al.*, 2010). It is expected that the ubiquitous presence of bioactive molecules like APIs in the aquatic ecosystems may have the potential to affect analogous pathways on aquatic organisms presenting similar target organs, tissues, cells or biomolecules as during humans and/or mammals therapeutic action (Fent *et al.*, 2006a). As mentioned before, invertebrates also possess biomolecules that can be affected by the presence of the selected APIs NSAID IBU and DCF (i.e. PGs and COX activity) (Section 1.10.1) and SSRI FLX (i.e. 5-HT) (Section 1.10.2) MoAs (Osada and Nomura, 1990; Fong, 1998; Gagné and Blaise 2003; Gagné *et al.*, 2007; 2011). As sessile filter-feeders with a relatively long life-span, mussel *M. galloprovincialis* is extremely exposed to all sorts of contaminants (including APIs) present in their habitats' surrounding waters. These features along with a wide geographic distribution render them the recognition as preferential environmental sentinel species (Cajaraville *et al.*, 2000; Porte *et al.*, 2006; Cravo *et al.*, 2009) and consequently their selection in the present thesis as an optimal species for APIs exposure essays. Mostly regarding to acute effects bioassays, very few other studies have included long-term single and mixture APIs exposure assays using environmental realistic exposure concentrations on potential biochemical, metabolic and physiological response alterations in mussels' species applying a multibiomarker approach, which greatly impairs the current knowledge for a more comprehensive ERA feasibility (Van der Oost, 2003). More so, if considering that APIs major therapeutic functions are based on its specific redox reactivity and oxidative stability (Harmon *et al.*, 2006; Martín-Díaz *et al.*, 2009a). Therefore, the present thesis included the assessment of antioxidant enzymes SOD, CAT, GR and Phase II GST activities and LPO levels (oxidative stress and related damage) (Section 1.7), AChE activity (neurotoxic effect) (Section 1.8) and ALP related to vitellogenin-like proteins (endocrine disruption effects) (Section 1.9) effects after two weeks single and mixture exposure to the aforementioned APIs and a classical contaminant Cu.

7.2.1 Effect of IBU exposure in *M. galloprovincialis* (Chapter 3)

IBU is one of the most sold over-the-counter NSAIDs worldwide consequently being one of the highest detected and ubiquitous APIs in aquatic ecosystems (Parolini *et al.*, 2009; Santos *et al.*, 2010). Moreover, IBU was also defined as a class IA "highest risk for the environment" compound (Besse and Garric, 2008). Based on the available literature for surface waters at the time of the exposure assay, it was decided to use an average IBU environmental realist concentration of 250 $ng.L^{-1}$ to assess its effects on mussels *M. galloprovincialis*

7.2.1.1 IBU and Oxidative stress

The results (Sections 3.10.3 and 3.11.1) confirm a tissue and time-specific alterations of antioxidant enzyme activities in mussels exposed to IBU. In IBU-exposed gills (Figure 3.1 and 3.3), it was particularly evident the increase of SOD activity during the first week followed by its recovery by the end of the exposure period. The activities of CAT, GR and GST activities concomitantly decreased particular after 15 days. Whereas for IBU-exposed digestive gland (Figure 3.4 and 3.6), all the enzymes (except GST) were significantly enhanced after the 3rd (except SOD) and 7th days recovering to basal levels by the end of the exposure. LPO levels were nevertheless elevated in both tissues, in which either directly related to SOD activity in gills or GR activity in digestive glands. However, the differential antioxidant activity responses were linked to specific tissue functions and its particular ability to respond to IBU-derived ROS increase. Therefore even though, exhibiting a strong SOD activity increase, gills response was not as effective to counteract the inhibitory effect exerted by IBU-derived ROS over all the other antioxidant enzymes than in digestive glands resulting on a much higher LPO damage (Figures 3.2 and 3.5) which justified this latter as the organ most important in ROS detoxification response and antioxidant system recovery. Moreover, the fact that GR activity decreased constantly during the exposure in gills, suggested that in this tissue, IBU seriously affects cells ability to maintain a homeostatic level between GSSG/GSH (Vernouillet *et al.*, 2010). This is further supported by Phase II GST activity concomitant inhibition tendency due to a lesser presence of its substrate GSH (which acts as oxidative-radicals scavenger and enzymatic co-factor) (Regoli, 1998) compromising its detoxification conjugation reactions. Consequently only in digestive glands, there was enough free-GSH (to enhance GR activity) to further counteract IBU-

induced ROS, which nevertheless was not totally effective to prevent the elevation of LPO levels.

Generally, it was hypothesized that since AA accumulation is related to an increase of ROS formation (Cocco *et al.*, 1999; Delaporte *et al.*, 2006), that the blockage by NSAIDs of COX pathway, one of major metabolic pathways of AA, may be potentiating IBU derived ROS induction and consequently LPO damage in exposed mussels. Since there were no antioxidant activities concerning IBU exposure the data was compared to other APIs exposure essays in mussels' species. Therefore, the transient SOD activity pattern was similar to that of zebra mussel *Dreissena polymorpha* whole body exposed to analgesic PAR (150 $ng.L^{-1}$) on the first 24 hours and progressive decrease to control levels over 96 hours, whereas CAT activity induction is slower and less pronounced (Parolini *et al.*, 2010) this was associated though to a possible faster size-dependent metabolism of this freshwater species.

The results were also in agreement with the outline of LPO levels in gills after one week-exposure of *M. galloprovincialis* to anticonvulsant CAR (100 $ng.L^{-1}$) and with LPO levels in digestive glands of the same species after exposed to 23 $\mu g.L^{-1}$ of paracetamol) and of *E. complanata* exposed to 0.4, 2 and 10 mM carbamazepine (Martín-Díaz *et al.*, 2009a, b; Solé *et al.*, 2010) (Table 3.2). Also as observed in this study, although in *M. galloprovincialis* exposed to CAR showed no noticeable alteration of CAT activity in gills, this enzyme was significantly inducted in digestive glands (Martín-Díaz *et al.*, 2009b) which also occurred after a 24 hours injection of BEZ and GEM (0.1 nmol/animal) (Canesi *et al.*, 2007) and after 3 $ng.L^{-1}$ of DL-propranolol (Franzellitti *et al.*, 2011).

It was concluded in this Chapter, that although the contribution of other factors subjacent to the experimental design should not be excluded (such as food absence during the experiment which might have influenced some basal enzymatic variations and CI% at the beginning and the end of the exposure period) that even at a low concentration NSAID IBU does exert transient oxidative stress in *M. galloprovincialis* tissues. This affected mostly gills through CAT and GR activities breakdown and higher membrane damage, notwithstanding digestive gland also showed the necessity of overall enhanced activities of selected enzymes. Finally, both tissues showed signs of recovery by the concomitant decrease of LPO levels to basal levels.

7.2.1.2 IBU and Endocrine Disruption

Concurrently, considering endocrine disruption associated to IBU exposure, the results (Section 3.10.4 and 3.11.2) showed much more uniformity than evidences of oxidative stress, with significant increment of ALP levels in both males and females' gonads, in particular by the 2-fold higher levels of Vtg-like proteins in exposed males after one week (Table 3.4). It was argued, that the induction of these proteins was an effective biomarker of feminization in mussel *E. complanata* exposed for 30 days to NSAIDs containing primary-treated municipal extract (including IBU at concentrations of $\mu g.L^{-1}$) (Gagné *et al.*, 2005). Even though, this author also confirmed alterations by the assessment of female/male ratio and the expression of a female-specific protein in males, the present results clearly indicate an effective IBU exposure impact causing endocrine disruption effects which potentially lead to mussels' reproductive fitness impairment or ultimately its feminization.

In summary, the exposure to IBU at an environment realistic concentration (250 $ng.L^{-1}$) beside inducing significant transitory antioxidant defense responses and LPO particularly in gills, indicated a more severe and continuous impact of IBU as an endocrine disruptor, particularly in males, with consistently higher Vtg-like proteins induction throughout the exposure period.

7.2.2 Effect of FLX exposure in mussel *M. galloprovincialis* (Chapter 4)

FLX is the highest prescribed antidepressant in the world. Acting as a SSRI it regulates the increase of 5-HT neurotransmitter levels in neurons (Hiemke and Härtter, 2000; Fent *et al.,* 2006). 5-HT is associated to the modulation of important functions in hormonal and neuronal mechanisms in both vertebrates and invertebrates (Fong, 2001; Fent *et al.*, 2006; Stanley, 2007; Painter *et al.*, 2009; Styrishave *et al.*, 2011) but also to affect antioxidant system in mice (Djordjevic *et al.*, 2011). Applying the same principle, FLX environmental realistic concentration was chosen after careful review of all literature relating its occurrence in surface waters (Table 4.1) and reported effects in non-target organisms (Table 4.2).

7.2.2.1 FLX and Oxidative Stress

Results (Section 4.3.2) revealed a transient antioxidant status alteration in both mussels' tissues after exposure to SSRI FLX (75 $ng.L^{-1}$), although with totally different activity profiles than that observed after IBU exposure. In both tissues, SOD activity (Figure 5.1A and B) was down regulated over time, although only significant in gills by the end of the exposure. Moreover in digestive gland SOD activity was inversely related to CAT activity. SOD activity down regulation in the tissue, was due to the elevation of ROS levels by FLX presence, particularly H_20_2. This oxygen-radical is the substrate for CAT activity responsible for the its conversion to less toxic compounds such as H_20 and 0_2 (Regoli and Principato, 1995), therefore as aforementioned for IBU, the preferential role of digestive glands on general redox-cycling (Livingstone *et al.*, 1992) justified CAT's activity swift trigger over the first 3 days of exposure, fluctuating again to less elevated levels by the end of the exposure. Although no alterations of CAT were ever mentioned in other FLX exposure essays, this is though in agreement to the confirmed increase of ROS production in rainbow trout (*Oncorhynchus mykiss*) hepatocytes and SOD activity inhibition in mice exposed to 5 $mg.kg^{-1}$ FLX body mass (Laville *et al.*, 2004; Djordjevic *et al.*, 2011). Higher CAT activities were also reported in *M. galloprovincialis* digestive glands than in gills either after IBU (as referred in the previous section) or CAR exposure (Martín-Díaz *et al.*, 2009b; Gonzalez-Rey and Bebianno, 2011; 2012). Moreover, the transitory outline of GST activity in both tissues (Figures 4.1E and F) which included some variations on the basal GST activities (pointing also to other variables affecting this enzyme activity outcome) showed a higher responsiveness of digestive gland and a direct relationship with CAT activity also observed after IBU, CAR, BEN and PRO exposures, relating to evidences of partial metabolization of FLX in this tissue (Canesi *et al.*, 2007; Martín-Díaz *et al.*, 2009b; Franzellitti *et al.*, 2011; Gonzalez-Rey and Bebianno, 2012) (section 1.2.1.1). Again, but at to a lesser extent than that observed in IBU exposure, FLX-exposed gills were again more prone to LPO damage (4-fold lower than after the first week of IBU exposure) (Figure 4.2A and B) and antioxidant system breakdown than digestive gland as highlighted by SOD activity and LPO levels related dispersal distribution throughout time opposing to the one of exposed-digestive gland time groups (Figure 4.6).

7.2.2.2 FLX and Neurotoxic Effect

It was hypothesized that since SSRI FLX MoA involves the increase of 5-HT concentration, as observed in *E. complanata* after injection with FLX (Gagné and Blaise, 2003), that AChE function over neurotransmitter ACh hydrolysis was also affected by FLX derived 5-HT induction. The results (Section 4.3.4 and 4.4.2) pointed out to an induction of AChE activity after the first three days only inhibited by the end of the exposure period. Usually, AChE activity is inhibited by APIs, as observed for *M. galloprovincialis* gills exposed to PAR (23 and 403 µg.L-1) and *E. complanata* visceral mass exposed to DIA (4, 20 and 100 nmol per mussel) (Solé *et al.*, 2010; Gagné *et al.*, 2011). Since very few other studies showed AChE induction in invertebrates, like mussels *E. complanata* from an aeration lagoon (Gagné *et al*., 2010), it was rather difficult to ascertain this phenomenon. Since AChE induction implies the depletion of ACh by an increased hydrolysis of this neurotransmitter to choline and acetic acid (Tsuchiya *et al.*, 2004), several hypotheses were given to explain AChE increase in mussels' gills tissue over the first days, such as the possible depletion of ACh due to its competition with 5-HT at the receptor activation level resulting on necessity of AChE activity further upregulation; an association of AChE increase due to cell apoptosis (as described by Zhang *et al*., 2002) or a link between AChE activity induction and low levels of E2 (Tsuchiya *et al.*, 2004).

7.2.2.3 FLX and Endocrine Disruption

Contrarily to what was observed after IBU exposure (showing the consistent upregulation of ALP levels particularly in males) (Chapter 3B - Gonzalez-Rey and Bebianno, 2012), ALP levels after FLX exposure were altered rather by a constant down regulation in both sex-differentiated gonads as in females over the first 3 days of exposure. Although inverse to the Vtg-like proteins induction expected after exposure to other endocrine disruptors (Porte *et al.*, 2006; Gagnaire *et al.*, 2009), these results also attest an actual FLX impact in mussel' reproductive fitness. The evidence of ALP levels inhibition was in agreement with that observed in *M. edulis* females exposed to North Sea crude oil (NSO) which also presented decreased of gonads development (Ortiz-Zarragoitia and Cajaraville, 2006). These authors assumed that ALP levels may be either indirectly associated with gonad development in this species or by a directly related antiestrogenic effect of PAHs and oils, and therefore 5-HT levels should be

taken in account since this neurotransmitter is known to control gonad development and reproduction (Gagné and Blaise, 2003).

Furthermore, the ALP down regulation in both FLX-exposed female and male gonads may be associated to the inverse relationships between Vtg synthesis and 5-HT levels and/or 5-HT and E2 levels as well as the observed 5-HT increase during active spawning period observed in *E. complanata* and E2 requirement to initiate vitellogenesis (Gagné and Blaise, 2003; Matozzo *et al.*, 2008) with the E2 levels decrease observed in mussel *D. polymorpha* after exposure to FLX (20 and 200 $ng.L^{-1}$) between spawning and after-spawning phases (Lazzara *et al.*, 2012). Therefore, even though the E2 levels were not measured, the aforementioned a link between AChE increase and low E2 levels can be hypothesized, considering the observed Vtg-like decrease in FLX-exposed gonads along with AChE induction. For this reason, a histological study of FLX-exposed gonads along with the assessment of E2, 5-HT and FLX concentrations in mussels' tissues would complement the assumptions on reproduction fitness alteration given by ALP levels decrease.

In summary, the exposure to an environmental realistic concentration of FLX although not producing alteration on mussels' condition index, induced tissue and time-specific transient antioxidant enzyme activities and LPO in *M. galloprovincialis* (particularly affecting gills tissue). Yet these oxidative stress effects were not as evident as the overall ALP down regulation in gonadal tissues suggesting a higher influence of FLX as an endocrine disruption rather than an oxidative stress inducer. Additionally, the influence of FLX as a SSRI on 5-HT levels also clearly altered AChE activity possibly resulting on cholinergic neurotransmission functions breakdown linked to either a competition at the level of receptor activation between neurotransmitters or with the possibility of higher cell apoptosis. A link was also hypothesized for AChE activity increase with the concomitant Vtg-like proteins decrease (implicating lower E2 levels). Finally, even though FLX presence in the water induce ecotoxicological effects in mussel *M. galloprovincialis* particularly affecting its reproduction fitness, the mechanisms of 5-HT receptor activation in mussels are still unknown (Gagné and Blaise, 2003) and should be further assessed.

7.2.3 Effect of NSAID DCF exposure in *M. galloprovincialis* (Chapter 5)

NSAID DCF is, along with IBU, one of the most frequently applied anti-inflammatory in human and veterinary practice. This APIs has been ubiquitously detected in surface waters worldwide (Table 5.1) being recently included in the list of priority substances under the European Commission (European Commission, 2012a, b). In a ecotoxicology perspective, DCF was one of the first APIs associated to cause serious impact in non-target animals, after being linked to the poisoning and liver failure of Indian vultures (*Gyps* sp.) after scavenging DFC-treated livestock carcasses (Oaks *et al.,* 2004; Saini *et al.*, 2012). As for the other APIs, DCF environmental realistic concentration applied in the exposure assay (250 $ng.L^{-1}$) was based on currently available literature reporting its occurrence in surface water.

7.2.3.1 DCF and Oxidative Stress

The results (Section 5.3.2 and 5.4.1) showed an overall higher basal antioxidant enzymes activity (except CAT) in gills than in digestive glands but also a clear cluster distribution of each tissue associated to these differences (Figure 5.6). The direct relationship of SOD and GR activities in gills with CAT activity and further LPO levels in digestive glands (Table 5.3) suggested different tissue function and transport on the counteraction of DCF-induced ROS increase. In this case, SOD and GR activity induction was firstly triggered in gills (and to a lesser extent in digestive glands) after 3 days of DCF exposure, thus resulting in a CAT activity induction in digestive glands to convert H_2O_2 produced by SOD into H_2O and O_2 (Van der Oost *et al.*, 2003; Viarengo *et al.*, 1995), nevertheless the parallel increase of free oxygen-radical scavenger GSH due to GR activity increase was not enough to prevent LPO in DCF-exposed digestive gland. However, the significant inhibition of antioxidant system activities and LPO levels in the tissues showed an antioxidant system recovery after the first days of DFC-induced oxidative stress effects by the end of the experiment. Moreover, even though GSH was available for the eventual phase II conjugation reaction by GST, this enzyme activity was not altered in exposed tissues showing a direct relationship with digestive gland-SOD rather than with GR or CAT activity (Figure 5.1G and H, Table 5.3). Therefore, these results showed that at this concentration DCF does not promote phase II detoxification contrasting with that showed by the concomitant induction of GST and LPO in *D. polymorpha* and *Mytilus spp.* exposed to 4-fold higher concentration of DCF

(Quinn *et al.*, 2011; Schmidt *et al.*, 2011). Therefore, even mussel tissues showed some significant alterations in antioxidant enzymes responses after the exposure to DCF, in terms of oxidative stress results were pertained as much more inconclusive and unclear than the ones observed in the previously exposure essays to IBU and FLX (Gonzalez-Rey and Bebianno, 2012; 2013 - Chapter 3 and 4).

7.2.3.2 DCF and Neurotoxic Effect

Like, observed after FLX exposure, DCF clearly induced AChE activity. In this case the increase was lower in proportion to basal levels but longer, extending also to the first week (Gonzalez-Rey and Bebianno, 2013 - Chapter 4). As mentioned, AChE increase was not expected and contrasted to that observed in mussels exposed to PAR and DIA (Solé *et al.*, 2010; Gagné *et al.*, 2011). Therefore as observed after FLX exposure (except the competition between ACh and 5-HT receptor activation), even though it is extremely difficult to ascertain the reason why AChE increased in DCF presence it was also hypothesized that after DCF exposure the AChE activity increase may be related to either possible cell apoptosis as suggested by Zhan *et al.* (2002) or due to its inverse relationship with endogenous E2 levels (which in turn are related to vitellogenesis) (Tsuchiya *et al.*, 2004; Matozzo *et al.*, 2008).

7.2.3.3 DCF and Endocrine disruption

The results (Section 5.3.5 and 5.4.2) only showed the induction of ALP levels in DCF-exposed females (after 3rd and 7th days) and in exposed-males (only after the 3rd day) (Figure 5.4). It was argued that the concomitant ALP levels increase in non-exposed mussels, particularly males, possibly overshadowed a clearer proof of endocrine disruption after DCF exposure. Even though, vitellogenesis occurred at the beginning of DCF exposure, again the exposure to IBU and FLX induced much stronger evidences of these APIs impact as endocrine disruptors than DCF (Gonzalez-Rey and Bebianno, 2012; 2013 – Chapter 3 and 4). These results are again in disagreement to that of the exposure to a 4-fold higher concentration of DCF, as no alterations of ALP levels exist in exposed-mussels *D. polymorpha* and *Mytilus spp* (Quinn *et al.*, 2011; Schmidt *et al.*, 2011). Moreover, though its significance may be less obvious than the possible connection between AChE activity increase and cell apoptosis, the direct relationship

between ALP levels in females and AChE induction (Table 5.3) also give evidence to an unknown and possible DFC-derived interference with mussels' estrogen receptors.

In summary, the highest effects of DCF exposure were related to the first 3 days of exposure, showing overall recovery responses by the end of the exposure period. Although the antioxidant system seemed to be more affected and altered in gills, the LPO levels were slightly more evident in digestive glands. Moreover, even though DCF-exposed gonads showed some evidence of ALP levels induction over the first week, the highest impact of DCF was further related to the neurotoxic effect associated to the clear AChE activity enhancement by a potential estrogenic activity alteration or cell apoptosis. This statement, however, can only be confirmed through the concomitant quantification and interactions assessment of E2, PGs and COX activity in mussel tissues.

7.2.4 Effect of APIs mixtures exposure in *M. galloprovincialis* (Chapter 6)

APIs occur in the aquatic environment as complex mixtures, as clearly demonstrated in Chapter 1 and 2. If the information lacks for single exposure APIs effects assessment it is even more elusive concerning environmental realistic concentrations APIs mixtures (Table 6.1). Therefore, it is crucial to assess the possible effects derived from the interactions between multiple stressors especially since they may differ to those associated to single exposures. Although it is difficult to mimic a totally realistic scenario, it was intended to assess the possible interactions between the selected APIs (MIX 1), and those introducing a common environmental contaminant Cu (MIX 2). For this an equivalent experimental design was performed using the same APIs concentrations and an equivalent multibiomarker approach as that described above concerning single selected APIs.

7.2.4.1 Mixtures and Oxidative Stress

First of all, neither mixture induced alterations in mussels' CI%. Nevertheless, the antioxidant enzyme activities and LPO levels were mixture, tissue and time-specific. While, on one hand Cu present in MIX 2 generally induced higher and more progressive LPO levels in gills by the concomitant inactivation or inhibition of SOD, CAT and GR activities than MIX 1. MIX 1 exposed-gills showed the same antioxidant enzymes as well as phase II GST enhancement by the end of the experiment rendering a more

effective protection against ROS induced LPO (Figure 6.2A). On the other hand, the fact that MIX 1- exposed digestive gland showed swifter antioxidant enzyme responses than gills counterparts consistently prevented this tissue from LPO damage, and confirmed this tissue as better ‘equipped’ to deal with this mixture ROS-induced detoxification. Again, Cu in MIX 2 promoted a general inactivation of most enzymes (except GR activity) in digestive glands. However the GR activity enhancement, freeing ROS-scavenger GSH in this tissue cells, was not enough to prevent the significant induction of LPO in this tissue by the end of the second week.

7.2.4.2 Mixtures and Neurotoxic effect

The results also showed (Figure 6.3) that neither one of the mixtures exerted significant changes in AChE activity. Although MIX 2-exposed gills showed generally higher AChE activity ($p > 0.05$), it was assumed that these mixtures do not promote neurotoxic effect in exposed mussels.

7.2.4.3 Mixtures and Endocrine disruption

Induction of ALP levels occurred in females exposed to both mixtures over the 3rd day and first week (Figure 6.4A). Nevertheless after the second week the concomitant enhancement of the basal levels as well as differential responses toward mixtures rendered the ALP levels outcome rather unclear. In males (Figure 6.4B) MIX 1 exerted a continuously impact on ALP levels than MIX 2. As mentioned for DCF-exposure (section 7.2.3) the fact that the basal levels varied with time might have masked the results. Nevertheless, both sex-differentiated gonads showed at some point the induction of ALP levels, giving evidence of these mixtures ability (particularly MIX 1) to induce endocrine disruption in exposed mussels.

7.2.4.4 Interaction between mixture components

As it is evident, there were several noticeable differences between each mixture and single APIs exposure effects on oxidative stress responses (antioxidant enzymes activities and LPO levels), neurotoxic effect and endocrine disruption.

The interaction between mixture components assessment for each biomarker is summarized in Table 7.1.

Table 7.1: Interaction between mixture components for each biomarker

Biomarkers/Tissue	Time (days)	IBU	FLX	DCF	Cu[a]	MIX 1	MIX 2	Possible Interactions
Oxidative stress								
SOD activity								
gills	3	↑*	↓	↑*	nd	↓	=	• MIX 1:FLX antagonistic effect with NSAIDs MIX 2: Cu counteracts FLX to basal levels
	7	↑*	↓	↓	↓	↓	↓	• MIX 1 and 2: Synergism between FLX, DCF with or without Cu antagonist with IBU
	15	↑	↓*	↓*	nd	↑*	↓	• MIX 1: Potentiation of IBU against FLX and DCF MIX 2: Cu cancels IBU independent action
digestive gland	3	↑	↓	↑	nd	↓	↑	• MIX 1: FLX antagonic effect with NSAIDs MIX 2: Cu antagonist with FLX
	7	↑*	↓	↑*	↓	↑*	↑	• MIX 1: NSAIDs potentiated, FLX has no effect MIX 2: Cu inhibits SOD activity although its activity is still slightly enhanced
	15	↑	↓	=	nd	=	↑	• MIX 1: Addictive effect APIs MIX 2: Synergetic effect between Cu and IBU potentiates slight increase
CAT activity								
gills	3	↓	↑	↓	nd	↓	↓	• MIX 1 and 2: Addictive effect between APIS with or without Cu
	7	↓	↑	↓	↑	=	=	• MIX 1 and 2: Synergetic effect of NSAIDs antagonistic with FLX and/or Cu
	15	↓*	↑	↓	nd	↑	↓*	• MIX 1: FLX antagonistic effect with NSAIDS MIX 2: Synergism between Cu and NSAIDs antagonist to FLX

Table 7.1: (Continuation).

CAT activity								
digestive gland	3	↑*	↑*	↑*	nd	↓	=	• MIX 1: Antagonistic effect between the three APIs MIX 2: Effect of Cu resulted and less pronounced antagonism between APIs
	7	↑*	↑	↓	=	↑*	↓	• MIX 1: Antagonism of DCF with the synergism between FLX and IBU MIX 2: Antagonistic effect of IBU and FLX against the potentiation of DCF effect by Cu
	15	↓	↑*	↓*	nd	=	↓	• MIX 1: Antagonism between FLX and NSAIDs • MIX 2: Addictive behavior between APIs and Cu
GR activity								
gills	3	↓*	nd	↑*	nd	↓	=	• MIX 1: Significant downregulation seemed to follow DCF effect (on the 3rd day) antagonistic with IBU • MIX 2: Antagonistic effect between IBU and remaining mixture components
	7	↓*	nd	=	↑	=	↓	
	15	↓*	nd	=	nd	↑*	=	
digestive gland	3	↓*	nd	↑	nd	↓	=	• MIX 1: Follow IBU effect antagonistic to DCF MIX 2: Cancelling of IBU effect by Cu
	7	↓*	nd	↑	↓	↑	↑*	• MIX 1: Follow DCF effect antagonistic to IBU MIX 2: Cu potentiated DCF effect cancelling IBU effect
	15	↑	nd	↓	nd	=	↑	• MIX 1: Antagonism between NSAIDs • MIX 2: Cu induced slight enhancement over APIs antagonistic effect

Table 7.1.: Continuation).

GST activity								
gills	3	↓	↑	↑	nd	↓	↑*	• MIX 1: Addictive effect between APIS MIX 2: Cu synergism with FLX and DCF and antagonism with IBU
	7	↓	↑*	↑	=	↑	↓	• MIX 1: Antagonism between addictive effect of FLX and DCF with IBU MIX 2: Addictive effect of APIs with Cu
	15	↓*	↓	↑	nd	↑*	=	• MIX 1: DCF potentiation (unaltered by neither IBU nor FLX) MIX 2: Cu blocked DCF potentiation
digestive gland	3	↑	↑	↑	nd	↓	=	• MIX 1 and 2: Antagonistic effects of mixture component, less pronounce by Cu
	7	↑	=	↑*	=	↑*	↑*	• MIX 1 and 2: Potentiation of NSAIDS effect, not affect by FLX, Cu had a slight antagonist effect (lower enhancement of GST activity)
	15	↓	↑*	↑	nd	↑	↑*	• MIX 1: Synergism between FLX and DCF not antagonized by IBU MIX 2: Addictive effect of Cu with FLX and DCF
Damage								
LPO levels								
gills	3	=	↑	↑	nd	↑	↑*	• MIX 1 and 2: Addictive effect between mixture components
	7	↑*	↑*	↓*	=	↑	↑*	• MIX 1: Antagonistic effect between NSAIDs following similar FLX effect MIX 2: Cu synergism with both IBU and FLX
	15	↑	↑	↑	nd	↑*	↑*	• MIX 1 and 2: Addictive effect between mixture components
digestive gland	3	↑*	=	↑*	nd	↑	=	• MIX 1 and 2: Do not follow addictive effect of mixture effects or independent action
	7	↑*	↑*	↓*	↑	=	=	• MIX 1 and 2: Antagonism between NSAIDs and either FLX or FLX and Cu
	15	↑	↑*	↓	nd	=	↑*	• MIX 1: Antagonistic effect between APIs MIX 2: Cu is antagonistic to IBU, more similar to addictive effect between FLX and DCF

Table 7.1: (Continuation).

Neurotoxic effect								•
AChE activity								
gills	3	**nd**	↑*	↑*	**nd**	↑	↑	• **FLX and DCF single exposure effects seem to lead to AChE induction tendency**
	7	**nd**	=	↑*	**nd**	↑	↑	
	15	**nd**	=	=	**nd**	↑	↑	
Endocrine disruption								
Vitellogenin-like proteins levels								
female gonads	3	↑	↓*	↑*	**nd**	↑*	↑*	• **MIX 1 and 2: Potentiation of NSAIDs effects antagonized by FLX and Cu**
	7	↑*	↓*	↑*	**nd**	↑*	↑*	• **MIX 1: Synergetic effect of NSAIDs antagonized by FLX** **MIX 2: Cu potentiates the synergism between NSAIDs**
	15	↑*	↓*	↑	**nd**	↑	=	• **MIX 1 and 2: DCF effect is antagonized by addictive effect of FLX and IBU, more evident by the presence of Cu**
male gonads	3	↑*	↓*	↑*	**nd**	↑	↑	• **MIX 1: Antagonistic effect of DFC and FLX with IBU** **MIX 2: Cu potentiated slightly the effect observed in MIX 1**
	7	↑*	↓*	↓	**nd**	↑	↓	• **MIX 1: Antagonism between IBU with FLX and DCF** **MIX 2: Antagonism between APIs addictive effects with Cu**
	15	↑*	↓*	↑	**nd**	↑*	=	• **MIX 1: Potentiation of APIs addictive effects** **MIX 2:Cu antagonized the effect observed for MIX 1**

* significantly different ($p < 0.05$); a - 5 µg Cu.L^{-1} (Maria and Bebianno, 2011); nd – not determined

7.3 Conclusions

These findings collectively proven that APIs are ubiquitous in all types of surface waters and promote relevant impact in species *M. galloprovincialis*. Therefore it becomes evident that to prevent further APIs environmental implications in wildlife it is absolutely necessary to review and update current WWTP processes, to follow a further implementation and abide of current EMEA and FDA environmental guidelines, but also to inform the general public, physicians and manufacturers about APIs problematic in the environment to assure an easier adoption of "Green Chemistry" strategies (FDA, 1998; Daughton, 2003; EMEA, 2006; 2007; EEA, 2010).

7.4 Future perspectives

Taking in account all the results presented in this thesis, further research should be performed to clarify and complement several aspects, overcome some limitations and enable a better comprehension related to APIs exposure in *Mytilus galloprovincialis*:

- Assessment of each API concentration in mussels' tissues and surrounding water, its bioaccumulation factor and preferential accumulation target-organ,
- Quantification of enzyme COX activity, phospholipid AA and eicosanoid PG concentration in mussels tissues to ascertain whether NSAIDs do promote the non-selective inhibition of COX 1 and 2 isoforms and associated metabolization pathways in mussels as they do in humans and mammals,
- Assessment of 5-HT in mussels exposed to SSRI fluoxetine to confirm the connection between FLX exposure and 5-HT levels enhancement,
- Histological study of sex-differentiated the gonads and E2 levels concentration assessment to further complement findings related to APIs-derived Vtg-like proteins levels alteration,
- Target cell apoptosis related enzymes or perform techniques associated to lysosomal membrane stability and permeabilization to ascertain possible linkage with AChE activity induction,
- Application of "exposomics" techniques (such as genomics, metabolomics, transcriptomics and proteomics) to ascertain each API metabolization pathways in invertebrates, alteration in proteins and gene profile expressions (proteome

and genome) due to APIs exposure and identification of more effective APIs exposure biomarkers.

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