UNIVERSIDADE DE LISBOA
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Pharmaceuticals in the environment: occurrence and exposure effects in non-target fish species

"Documento Definitivo"

Doutoramento em Biologia
Especialidade de Fisiologia e Bioquímica

Irina Almeida Duarte

Tese orientada por:
Professora Doutora Vanessa Fonseca
Professor Doutor Henrique Cabral
Professor Doutor Jerker Fick

Documento especialmente elaborado para a obtenção do grau de doutor

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DECLARATION

According to the paragraph 2a) of the Article 26 of the Regulation of the Post-graduate Studies of the University of Lisbon (Diário da Républica Nº 175 de 2020, 2ª série de 8 de setembro de 2020), this thesis includes a compilation of scientific papers published in collaboration with other co-authors.

This thesis is comprised by the papers corresponding to Chapters 2 to 5, of which the candidate is the leading author, and was responsible for conceptualization, methodology (sample collection and processing, laboratory analytical procedures), formal analysis (data and statistical analysis), and manuscript writing of all the papers. The four papers have been published in international peer-reviewed journals:

Chapter 2

Bioconcentration of neuroactive pharmaceuticals in fish: Relation to lipophilicity, experimental design and toxicity in the aquatic environment

Duarte I.A.; Fick J.; Cabral H.N.; Fonseca V.F.

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Chapter 3

Neuroactive pharmaceuticals in estuaries: occurrence and tissue-specific bioaccumulation in multiple fish species

Duarte I.A.; Reis-Santos, P.; Fick, J.; Cabral, H.N.; Duarte, B.; Fonseca, V.F.

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Chapter 4

Biomarker and behavioural responses of an estuarine fish following acute exposure to fluoxetine

Duarte I.A.; Pais, M.P.; Reis-Santos, P.; Cabral, H.N.; Fonseca, V.F.

Published in *Marine Environmental Research* (2019) 147: 24-31.

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Chapter 5

Depressed, hypertense and sore: Long-term effects of fluoxetine, propranolol and diclofenac exposure in a top predator fish

Duarte I.A., Reis-Santos P.; Novais S.C.; Rato, L.D.; Lemos M.F.L.; Freitas A.; Pouca, A.S.V.; Barbosa J.; Cabral H.N.; Fonseca V.F.

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ABSTRACT

Pharmaceutical contamination in coastal ecosystems is an emerging environmental issue, with neuroactive pharmaceuticals of particular concern as they bioaccumulate in non-target fish, affect the central nervous system and can trigger population-level effects. The presented outcomes disclose recent research efforts, revealing multiple adverse effects of exposure to neuroactive pharmaceuticals in fish, albeit skewed data concerning few neuroactive compounds and largely freshwater species exist. Moreover, bioconcentration is seldom considered and rarely determined in combination with other endpoints, hampering the link between internal dosage and effects. Also, estimating the bioconcentration of neuroactive pharmaceuticals through lipophilicity is not straightforward, depending on multiple experimental factors. Here, nine neuroactive compounds were signalled as potentially threatening in aquatic ecosystems due to environmental concentrations either exceeding or near thresholds known to significantly affect fish behaviour, growth and condition or reproduction. Up to 28 neuroactive pharmaceuticals were detected in estuarine surface waters and seven fish species demonstrating the diversity and pervasiveness of neuroactive compounds in both high and slightly impacted coastal ecosystems. Bioaccumulation among all species revealed no clear pattern linked to compounds lipophilicity, species habitat use or trophic level, with higher frequency and concentrations observed in the brain, followed by liver and muscle tissues. Acute and chronic exposure experiments with two estuarine/marine fish species evidenced the toxicity of three pharmaceuticals with different modes-of-action, highlighting higher uptake and toxicity of the neuroactive compound fluoxetine in comparison to other frequently detected compounds tested. Sub-individual measurements revealed effects on critical processes (e.g. antioxidant and biotransformation mechanisms, or energetic metabolism), whereas individual-level effects of higher ecological relevance (e.g. alterations to growth, feeding or activity behaviours) followed chronic exposure or acute exposure at higher concentrations. Overall, critical insights on environmental fate and exposure effects in fish are provided, highlighting the need for priority research and continuous monitoring of neuroactive pharmaceuticals in coastal ecosystems.

KEYWORDS: Coastal pollution, Neuroactive pharmaceuticals, Estuarine and marine fish, Bioaccumulation, Toxicity

RESUMO

A contaminação por fármacos nas zonas costeiras constitui um problema ambiental emergente, onde os fármacos neuroativos são de particular importância porque bioacumulam em espécies não-alvo, afetando o sistema nervoso central e causando efeitos ao nível das populações. Os resultados demonstram, na literatura existente, os diversos efeitos adversos da exposição de fármacos neuroativos em peixes, apesar de incidir sobre poucos compostos e maioritariamente espécies de água-doce. A bioconcentração é também insuficientemente considerada e raramente estudada em combinação com outras respostas biológicas, dificultando a conjugação entre concentração interna e efeitos observados. A estimativa da bioconcentração dos fármacos neuroativos através da sua lipofilicidade não é direta e depende de múltiplos fatores experimentais, o que dificulta a previsão de risco. Contudo, concentrações ambientais de nove fármacos neuroativos excedem ou estão próximas de concentrações que causam efeitos deletérios em peixes. Dados ambientais, com a deteção de até 28 fármacos neuroativos em águas superficiais e peixes, evidenciam a ubiquidade e diversidade destes compostos em estuários, sendo que o padrão de bioacumulação em sete espécies de peixes foi independente da lipofilicidade dos compostos, do uso do habitat ou do nível-trófico das espécies, com maior frequência e concentrações observadas no cérebro, seguido do fígado e músculo. Experiências de curta e longa exposição a fármacos neuroativos com duas espécies estuarinas/marinhas demonstraram a toxicidade de fármacos com diferentes modos-de-ação, revelando maior acumulação e toxicidade do fármaco neuroativo, fluoxetina, em comparação com outros fármacos frequentemente detetados. As respostas sub-individuais revelaram efeitos em processos essenciais (e.g. mecanismos antioxidantes, biotransformação, metabolismo energético), enquanto efeitos individuais de relevância ecológica (e.g. crescimento, comportamentos alimentares e locomotores) ocorreram após exposição crónica, ou aguda a concentrações mais elevadas. Em suma, novos conhecimentos relativos à presença, acumulação e efeitos da exposição em peixes, demonstram a necessidade de priorizar a investigação e monitorização dos fármacos neuroativos em ecossistemas costeiros.

PALAVRAS-CHAVE: Poluição costeira, Fármacos neuroativos, Peixes estuarinos e marinhos, Bioacumulação, Toxicidade

RESUMO ALARGADO

A ocupação das áreas costeiras compreende atualmente aproximadamente metade da população mundial, potenciada pela oferta de recursos e serviços dos ecossistemas estuarinos e marinhos. Desta forma, os estuários são alvo de múltiplas ameaças, associadas às várias atividades humanas, entre as quais a introdução de contaminantes nos ecossistemas costeiros, afetando os organismos aquáticos e a produtividade e qualidade destes ecossistemas. Os fármacos constituem um grupo complexo de compostos com propriedades físico-químicas diversas e com vários modos de ação, cuja utilização tem enorme relevância na melhoria da qualidade de vida do Homem, auxiliando no tratamento e prevenção de inúmeras doenças, bem como em contexto veterinário. Os fármacos são utilizados globalmente e em grandes quantidades, e o seu consumo tem vindo a aumentar nas últimas décadas, tendência que se prevê acompanhar o crescimento populacional, e maior necessidade e facilidade de acesso. Após o seu consumo, uma porção destes compostos é excretada pelo organismo, podendo ser encaminhada para estações de tratamento de águas onde, contudo, a sua eliminação é frequentemente incompleta, resultando na sua libertação para os ecossistemas aquáticos. Esta constante descarga constitui um problema emergente, uma vez que os fármacos são biologicamente ativos a concentrações muito reduzidas, e atuam em vias/alvos moleculares que são comuns entre o Homem e outros organismos, em particular em vertebrados como os peixes. Assim, os fármacos são considerados contaminantes emergentes, potencialmente nocivos para o ambiente aquático e para a saúde humana. Em particular, os fármacos neuroativos, que atuam no sistema nervoso central, são potencialmente prejudiciais ao interferirem com processos essenciais que regem o funcionamento do cérebro, alterando comportamentos essenciais como locomoção, alimentação ou reprodução, que em última análise culminem em alterações ao nível das populações. Estudos recentes têm demonstrado a toxicidade dos fármacos neuroativos, sendo reconhecidos efeitos em várias espécies não-alvo, incluindo invertebrados e peixes, existindo, no entanto, uma lacuna de conhecimento relativamente à ocorrência e efeitos em ambientes estuarinos e marinhos.

Neste contexto, o objetivo principal deste trabalho é estudar a presença e bioacumulação de fármacos neuroativos em ecossistemas estuarinos, bem como investigar os efeitos de exposição aguda e crónica em espécies de peixe estuarinas e marinhas, contribuindo desta forma para uma melhor avaliação do risco ambiental destes compostos.

A presente tese é composta por seis capítulos, quatro dos quais constituem artigos científicos publicados em revistas indexadas, sendo estes precedidos por um capítulo de introdução

geral ao tema (capítulo 1) e seguidos de um capítulo final de discussão geral e considerações finais (capítulo 6).

O primeiro capítulo consiste num enquadramento geral ao tema da tese, com foco na contaminação ambiental por fármacos e respetivas implicações. São abordadas várias questões relativas ao tema, nomeadamente a origem dos fármacos no ambiente e a sua classificação como poluentes emergentes; a contaminação em ecossistemas estuarinos, zonas altamente urbanizadas; o potencial nefasto da presença e acumulação de fármacos neuroativos em particular; a importância da utilização de espécies de peixes como indicadores da qualidade do habitat e a utilização de biomarcadores como ferramentas de análise de respostas à exposição a fármacos.

No capítulo 2 são explorados os mais recentes desenvolvimentos presentes na literatura sobre fármacos neuroativos, através de uma revisão sistemática abrangendo a bioconcentração e efeitos de exposição em peixes. O trabalho desenvolvido permitiu explorar vários padrões e limitações no conhecimento da toxicologia dos fármacos neuroativos. Em particular, os resultados revelam uma tendência de investigação limitada a apenas alguns fármacos e bastante centrada em espécies de água doce, comparativamente a espécies estuarinas e marinhas. De entre as respostas analisadas, a bioconcentração é insuficientemente considerada e raramente em combinação com outras respostas biológicas, dificultando a integração dos níveis de concentração interna com os efeitos observados. O estudo da relação entre a bioconcentração de fármacos neuroativos e a sua lipofilicidade, revelou que a sua utilização como indicador do potencial de bioconcentração não é imediata, e a análise dos dados existentes revela que a bioconcentração está, em parte, subordinada a fatores experimentais, abióticos e bióticos. A relação entre o potencial de bioacumulação e os efeitos de toxicidade observados (mortalidade, crescimento e condição, comportamento e reprodução) revelaram a incerteza da relação, à exceção da mortalidade, provavelmente associada à variabilidade e escassez da informação existente. Considerando as concentrações ambientais atuais, foram sinalizados nove fármacos neuroativos com maior potencial de risco, cujas concentrações nos sistemas aquáticos excedem, ou estão próximas de atingir, concentrações que causam efeitos significativos no comportamento, crescimento/condição ou reprodução dos peixes. No entanto, para alguns fármacos neuroativos não existe informação disponível, em particular em sistemas estuarinos e marinhos, realçando a necessidade de monitorizar e implementar estratégias de gestão de risco destes fármacos.

No capítulo 3 foi estudada a ocorrência e bioacumulação de 33 fármacos neuroativos em águas superficiais e em sete espécies de peixes estuarinas e marinhas de quatro ecossistemas

estuarinos com diferentes níveis de impactos. Os resultados revelaram a ubiquidade e diversidade de fármacos neuroativos presentes em águas superficiais, incluindo 28 fármacos pertencentes a diferentes classes terapêuticas como antidepressivos, antiepiléticos, psicostimulantes, opioides ou ansiolíticos, nos diferentes estuários, independentemente do nível de urbanização ou hidromorfologia dos mesmos. A bioacumulação de 13 fármacos neuroativos nas diferentes espécies de peixes revelou um padrão comum entre espécies e em todos os estuários, com maior frequência de deteção e concentrações no cérebro, seguido do fígado e do músculo. A bioacumulação de fármacos neuroativos foi observada em todas as espécies estudadas, não existindo, contudo, uma relação significativa com a lipofilicidade dos fármacos, o uso do habitat ou o nível trófico das espécies, apesar dos níveis acumulados variarem entre espécies. Aqui são revelados padrões de ocorrência e bioacumulação essenciais para a aplicação futura em estudos de análise de risco ambiental de fármacos neuroativos, e demonstrada a necessidade de investigar os impactos destes compostos em ambientes estuarinos.

No capítulo 4, foi estudada a toxicidade do antidepressivo fluoxetina através da exposição aguda de uma espécie estuarina residente, o caboz-comum *Pomatoschistus microps*, a concentrações ambientalmente relevantes e a concentrações mais elevadas. As respostas biológicas analisadas incluíram a atividade de enzimas antioxidantes, de biotransformação e de neurotransmissão, bem como danos de exposição ao nível dos lípidos e DNA, e alterações comportamentais (atividade locomotora e alimentação). O antidepressivo fluoxetina revelou-se capaz de interferir no funcionamento de mecanismos antioxidantes (e.g. inibição) e de biotransformação (resposta hormética) a concentrações ambientais, não tendo, contudo, incitado efeitos ao nível da neurotransmissão, de danos nos lípidos ou DNA, ou comportamentais, revelando uma certa tolerância desta espécie estuarina em comparação com estudos anteriores em espécies de água-doce. Contudo, a exposição a concentrações mais elevadas, revelou o impacto significativo deste antidepressivo no sistema colinérgico (neurotransmissão) e no comportamento locomotor e alimentar, cuja relação merece uma investigação mais aprofundada. Assim, torna-se evidente o potencial tóxico deste fármaco neuroativo, com potenciais implicações em comportamentos essenciais à aptidão e sobrevivência destes indivíduos, culminando em perturbações de elevada relevância ecológica.

No capítulo 5 é dado ênfase aos efeitos da exposição crónica de três fármacos com diferentes modos de ação, nomeadamente o antidepressivo fluoxetina, o anti-hipertensivo propranolol e o anti-inflamatório não esteroide diclofenac, com o objetivo de avaliar diferenças de toxicidade em fármacos frequentemente consumidos e presentes no ambiente. Para tal, juvenis de corvina *Argyrosomus regius*, foram expostos a concentrações ambientalmente relevantes dos

três compostos, tendo sido posteriormente avaliadas múltiplas respostas biológicas. Ao nível sub-individual foram analisadas alterações de mecanismos antioxidantes, de biotransformação, de neurotransmissão, de metabolismo energético e ainda a existência de danos oxidativos. Ao nível individual foram avaliadas alterações no crescimento e condição dos juvenis bem como a bioconcentração dos três fármacos nos tecidos musculares. Os resultados obtidos revelaram diferentes mecanismos afetados e diferentes níveis de toxicidade dos três compostos, sendo que o fármaco neuroativo (fluoxetina) mostrou maior bioconcentração e toxicidade que os restantes. A exposição à fluoxetina evidenciou uma extensa acumulação nos tecidos, resultando no decréscimo da taxa de crescimento dos juvenis de corvina, na ativação de defesas antioxidantes, na inibição de enzimas de biotransformação e no aumento de danos oxidativos nos lípidos e DNA no fígado. O anti-hipertensivo propranolol revelou uma toxicidade intermédia, com menor concentração nos tecidos que o fármaco neuroativo, causando, no entanto, o aumento de danos no DNA e reduzindo o metabolismo aeróbico no músculo, provavelmente como resposta ao aumento do stress oxidativo. Por último, o anti-inflamatório diclofenac não acumulou no tecido muscular, e mostrou o menor nível de toxicidade dos três compostos, todavia levando ao aumento do consumo energético celular no músculo e consequente redução da energia disponível.

Nestes capítulos experimentais (4 e 5) é demonstrada a importância de estudos que combinam respostas ao nível sub-individual e individual, permitindo conhecer os efeitos precoces e os mecanismos afetados, comparando diferentes níveis de toxicidade, e dando enfase à importância de integração de respostas ecologicamente relevantes.

No capítulo 6 é apresentada uma discussão geral dos resultados obtidos e perspetivas futuras de investigação, destacando a contribuição do presente trabalho com novos conhecimentos relevantes sobre a presença, bioacumulação e efeitos dos fármacos neuroativos em ambiente estuarino, e implicações para estudos futuros de avaliação de risco ambiental, enfatizando a necessidade de priorizar a investigação e a monitorização destes compostos em ecossistemas estuarinos e marinhos.

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ABBREVIATIONS, SYMBOLS AND UNITS

AChE	Acetylcholinesterase
ADHD	Attention Deficit Hyperactivity Disorder
ATC	Anatomical Therapeutical Chemical classification system
BAF	Bioaccumulation factor
BCF	Bioconcentration factor
BHT	Butylated hydroxytoluene
CAT	Catalase
CBH	Carbohydrates
CDNB	1-chloro-2,4-dinitrobenzene
CEA	Cellular Energy Allocation
cm	Centimetre
CYP	Cytochrome P450
DCF	Diclofenac
DF	Detection Frequency
DNA	Deoxyribonucleic acid
DNAd	DNA damage
DTNB	5,5’-dithio-bis(2-nitrobenzoic acid)
DTT	Dithiothreitol
dw	Dry weight
Ea	Energy, available
Ec	Energy, consumption
ECHA	European Chemicals Agency
EDTA	Ethylenediaminetetraacetic acid
EMA	European Medicines Agency
ER	Estuarine Resident species
EROD	Ethoxyresorufin O-deethylase
ETS	Electron Transport System
EU	European Union
FLX	Fluoxetine
G	Specific Growth rate
g	Gravitational force
g	Gram

GSH	Glutathione
GST	Glutathione S-transferase
HCl	Hydrochloric acid
IDH	Isocitrate dehydrogenase
IQR	Interquartile range
K	Fulton's condition factor
K_2HPO_4	Monobasic potassium phosphate
KCl	Potassium chloride
KH_2PO_4	Dibasic potassium phosphate
K_{ow}	Octanol/water partition coefficient
L	Litre
LC50	Lethal Concentration 50
LC-MS/MS	Liquid Chromatography-tandem Mass Spectrometry
LDH	Lactate dehydrogenase
Log	Logarithm
LOQ	Limit of Quantification
LP	Lipids
LPO	Lipid Peroxidation
Lt	Length
m/z	Mass to charge ratio
Max	Maximum
Med	Median
mg	Milligram
Min	Minimum
min	Minutes
mL	Millilitre
MM	Marine Migrant species
mM	Millimolar
MOA	Mode of action
MRC	Minimum Response Concentration
MS	Marine Straggler species
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate

NaOH	Sodium Hydroxide
ng	Nanogram
nmol	Nanomole
NSAID	Nonsteroidal Anti-inflammatory Drugs
OECD	Organisation for Economic Co-operation and Development
PCA	Principal Component Analysis
PMSF	Phenylmethylsulfonyl fluoride
PROP	Propranolol
PT	Proteins
PVDF	Polyvinylidene Fluoride
ROS	Reactive Oxygen Species
rpm	Rotations per minute
s	Seconds
SDS	Sodium Dodecyl Sulfate
SNRI	Serotonin and Norepinephrine Reuptake Inhibitors
SOD	Superoxide dismutase
SSRI	Selective Serotonin Reuptake Inhibitors
t	Time
TBA	2-Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substances
TCA	Trichloroacetic acid
TL	Trophic Level
TNB	Trinitrobenzene
U	Unit
UHPLC-TOF-MS	Ultra-High Performance Liquid Chromatography Time-of-Flight Mass Spectrometry
WHO	World Health Organization
WoS	Web of Science
Wt	Weight
ww	Wet weight
µg	Microgram
µL	Microlitre
ρmol	Picomole

Chapter 1

General introduction

Aims and importance of this thesis

Thesis outline

General introduction

Our oceans are experiencing broad and local scale impacts of human presence and activities. Humans have historically taken advantage of the natural resources and services offered by marine and coastal ecosystems, with almost half of the world's population settled in the vicinity of coastal areas (Martínez et al., 2007). As a result, estuaries are recognized as one of the most impacted ecosystems in the world due to urban development and industrialization, facing numerous anthropogenic impacts of varied nature (Halpern et al., 2015; Wolanski et al., 2019). Human activities linked to resources exploitation (e.g. fisheries, aquaculture), and destruction of habitats (e.g. dredging, land claim) or industrial and urban development, have contributed to significant habitat degradation in coastal environments at an increasing pace over recent decades, compromising ecosystems' health, with consequences for marine life (Cabral et al., 2022; Halpern et al., 2019). In particular, the input of land-based pollutants into aquatic systems, i.e., the drainage of chemical contaminants, nutrients and organic contents is of major concern. The majority of marine pollutants (80%) is estimated to originate from rivers' runoff and direct discharges of human-related activities, threatening marine organisms and affecting the productivity and quality of these ecosystems (Cabral et al., 2022; Wilhelmsson et al., 2013). Though it was generally thought that world's rivers, estuaries and oceans would carry away and dilute those discharges, the presence of hazardous substances that were previously undetected in complex environmental matrices (e.g. water, biota, soil) is now being disclosed by cutting-edge scientific research (Fatta-Kassinos et al., 2011). These newly noticed contaminants, for which toxicity has only recently been recognized, are referred to as contaminants of emerging concern (CEC) and include typically unregulated compounds that despite being released for many years, detection and quantification in the environment are fairly new (e.g. biocides, pharmaceuticals and personal care products). These compounds may profoundly affect aquatic wildlife, yet insufficient information concerning occurrence, fate and impacts in the environment exist, which results in their inclusion in monitoring programs (e.g. European Commission, 2022) meant to further address their persistence in the environment as well as their bioaccumulation and toxicity potential.

Over the last two decades, focus has been directed to the continuous release of human and veterinary pharmaceuticals into the aquatic environment (Daughton, 2016). Pharmaceuticals have long been produced and prescribed to improve human and animal health, lessening the impacts of illnesses and their symptomatology, and overall improving well-being. Notably, these compounds are consumed globally and in large quantities, with increasing trends in use

reported for decades, mostly reflecting the need for treatment of age-related and chronical diseases (OECD, 2021), and expected to continue increasing, linked to population growth allied to higher demand and access (Arnold et al., 2014; Bernhardt et al., 2017). After pharmaceutical consumption, a considerable portion of the compound is excreted from the human or animal body in its unchanged form (parent compound), or in more easily excreted forms, such as metabolized or conjugated forms (phase 1 and phase 2 metabolites) (e.g. Monteiro and Boxall, 2010; Patel et al., 2019). Once excreted, these compounds can be directly released, or flow through sewage systems, to be collected and treated in wastewater treatment plants (WWTP) before being released into the aquatic environment (aus der Beek et al., 2016; Wilkinson et al., 2022).

Pharmaceuticals consist of a diverse group of complex chemical compounds, of varied physicochemical and therapeutic properties, yet they share some key features that have led to their classification of emerging pollutants of priority concern (European Commission, 2013). Pharmaceuticals are biologically active at low concentrations, meaning that these compounds will potentially cause a biological response at environmentally relevant concentrations (ng/L – µg/L) (e.g. Corcoran et al., 2010; Fabbri, 2015), by targeting specific molecular pathways, which are in most cases evolutionary conserved, especially among vertebrates (Gunnarsson et al., 2008). Hence, when present in the environment, pharmaceuticals may trigger effects in non-target species, i.e. wildlife species other than human or target animals to which pharmaceuticals were meant to be applied. In this context, questions concerning the potential impacts of the presence of pharmaceuticals and personal care products (PPCPs) towards humans and wildlife were being published in the late nineties (Christensen, 1998; Daughton and Ternes, 1999), including on prescribed and unprescribed pharmaceuticals, but also fragrances, sunscreens or cosmetics, among others. Yet, only recently the detrimental impacts of pharmaceuticals in the environment are recognized, and the lack of information concerning their occurrence and potential impacts acknowledged.

Major sources of pharmaceuticals' input into the environment include wastewater treatment plants that receive influents from domestic, hospital and industrial sewage systems; the use of pharmaceuticals in animal production sectors, including aquaculture; improper disposal of unused or expired medicines; or indirectly using sludge from treatment plants to improve soil fertility (Arnold et al., 2014; Caldwell, 2016; Kümmerer, 2010; Patel et al., 2019). Higher concentration discharge of pharmaceuticals is mostly associated with manufacturing/production processes (up to mg/L range) (e.g. Fick et al., 2009; Larsson, 2014). However, the most prominent and consistent input of pharmaceuticals into the environment is through treated or

untreated wastewater discharges, where concentrations in the ng/L-µg/L range are typically reported. Conventional wastewater treatment systems use activated sludge processes that were primarily designed to remove pathogens, organic and inorganic matter in suspension from wastewaters. Hence, the elimination of specific compounds such as pharmaceuticals was generally beyond the scope of WWTP and thus removal efficiencies are highly variable and generally low for the majority of pharmaceuticals (e.g. Jelić et al., 2012; Rivera-Utrilla et al., 2013). Advances in tertiary wastewater treatments such as chlorination, ozonation, or membrane filtration can highly reduce the concentrations of some pharmaceutical compounds, despite low removal rates are still described in some cases (e.g. Adeleye et al., 2022). Overall, the persistent loads of pharmaceuticals result in exceptionally high concentrations of pharmaceuticals in WWTP, limiting its complete removal, resulting in residual concentrations still detected in final effluents (e.g. Loos et al., 2013), even for compounds with generally high removal rates. Moreover, pharmaceuticals are discharged on a continuous daily basis, contributing for its ceaseless input into aquatic systems, rendering them the classification of pseudo-persistent pollutants (Daughton and Ternes, 1999).

The presence of pharmaceuticals in the environment is still largely unregulated, given that public health is a priority, coupled to a lack of risk assessment data for compounds that entered the market before any legislation was applicable (Kuster and Adler, 2014), and the inexistence of regulation defining safety concentrations in the environment, a result of yet insufficient available information. In this context, many efforts to prioritize and improve risk assessment of pharmaceuticals have been made (e.g. Ågerstrand et al., 2015; Burns et al., 2018; Zhou et al., 2019), and currently regulatory actions account for pharmaceuticals as substances of priority concern that may constitute a risk for non-target species. The inclusion of pharmaceuticals in European legislation, namely through the European surface water Watch List, was proposed within the scope of the Water Framework Directive (WFD) objectives (European Commission, 2000). This Watch List includes substances signalled as potentially threatening to the environment and thus set for broad-scale monitoring in EU surface waters, including several pharmaceuticals and their metabolites, such as antibiotics, antifungals, and antidepressants (European Commission, 2022). However, much more research is needed to fully acknowledge the fate and impacts of pharmaceuticals in the environment and to unravel both bioaccumulation and toxicity potential in wildlife.

With thousands of pharmaceutical compounds currently marketed (Arnold et al., 2014; Arpin-Pont et al., 2016), over 600 compounds have already been detected in the environment all around the globe (aus der Beek et al., 2016; Wilkinson et al., 2022), including in highly

remote places such as the polar regions (e.g. Duarte et al., 2021; Kallenborn et al., 2018). Freshwater systems are most frequently screened for pharmaceutical presence, whereas estuarine and coastal areas are comparatively less studied, likely due to the expected dispersion and dilution processes (Fonseca and Reis-Santos, 2019; Gaw et al., 2014), and only a decade ago has attention been directed to these systems (Fabbri and Franzellitti, 2016; Gaw et al., 2014). Notwithstanding, these are critical receptors of both river basin inputs and direct inputs of urban wastewater discharges, and thus worthy of further investigation (Gaw et al., 2014). In fact, baseline studies report high concentrations of a large suite of pharmaceuticals in estuaries and coastal areas (e.g. Reis-Santos et al., 2018).

Among the different classes of pharmaceuticals that exist, neuroactive pharmaceuticals such as antidepressants, antiepileptics, anxiolytics are of particular concern. These compounds are designed to cross the blood-brain barrier and target the central nervous system, through different modes of action, interfering with essential chemical signalling processes that underlie brain functioning, thus ultimately likely to trigger population-level effects by altering, for instance, behavioural or reproductive endpoints (Brodin et al., 2014; Calisto and Esteves, 2009). Recent studies have underpinned the potential toxicity of neuroactive pharmaceuticals towards non-target species: effects have been reported in multiple species, including invertebrate and vertebrate organisms and include a variety of effects such as altered growth and development, reproduction and behavioural responses (e.g. Calisto and Esteves, 2009; Cunha et al., 2019, 2017; Sehonova et al., 2018). By sharing a high percentage of pharmaceutical targets with humans, due to conserved orthology of drug targets (e.g. *Danio rerio* over 90.0 %) (Gunnarsson et al., 2019), fish can be particularly susceptible to pharmaceuticals. Fish are frequently used as model species and biological indicators of habitat quality assessment in estuaries (Cabral et al., 2012; Deegan et al., 1997; Whitfield and Harrison, 2014), due to their wide distribution and long life cycle, their important ecological role as well as their economic value, among other factors (Cabral et al., 2022). Fish responses are key adaptations to environmental changes, both of anthropogenic or natural origin, and can be observed at different levels of biological organization (van der Oost et al., 2003). Early-warning signs, usually referred to as biomarkers, include any measurable alteration that arises from the interaction of a biological system with any environmental stressor, whether physical, chemical or biological (WHO, 1993). Biomarkers include sub-individual (e.g. biochemical, cellular or tissue) or individual (e.g. behaviour, development) alterations that can signal exposure, effects or susceptibility to any stressor, and are well recognized by the scientific community as a preeminent contribution/tool (van der Oost et al., 2003), by means of sensitivity and ecological relevance, in comparison with commonly used

endpoints in toxicity assessments such as mortality. At the sub-individual level, biochemical changes including the engagement of antioxidant defences or metabolic enzymes, as well as the oxidative stress revealed by lipid and DNA damage, are a few examples of early responses that can pinpoint exposure to hazardous contaminants. At the individual level, changes in individuals' growth, condition or alterations to behaviours such as feeding, locomotion or social interactions, are highly relevant as they evidence potential implications in terms of survival and population structure (e.g. Hamilton et al., 2016).

Likewise, the uptake of chemical contaminants is an important biomarker of exposure and an hazard measurement of toxicity, ultimately confirming the interaction with specific contaminants (McCarty et al., 2011; van der Oost et al., 2003). Pharmaceuticals are no exception, and bioconcentration in multiple tissues of organisms has been confirmed in laboratory and field studies (e.g. Duarte et al., 2022; Świacka et al., 2022). Determining the accumulation of different neuroactive pharmaceuticals in fish collected from the field, and in individuals exposed in the laboratory are key practices to investigate which compounds are more likely to bioaccumulate and cause detrimental effects, as well as to underpin risk assessment analysis (e.g. Fonseca et al., 2021). Bioaccumulation of lipophilic chemicals is usually estimated through each compound's physical and chemical properties, particularly through lipophilicity ($\log K_{ow}$), a measure of partitioning between polar (aqueous) and non-polar (octanol, tissue-like) fractions, which is currently used under European guidelines as a threshold for increased bioconcentration and toxicity potentials of chemicals and pharmaceuticals (ECHA, 2003; EMA, 2006). Yet, predicting pharmaceuticals' bioaccumulation through lipophilicity-based approaches has shown some inconsistencies, resulting in poor estimations of bioconcentration for some pharmaceuticals (e.g. Fick et al., 2010), and the suitability of such an approach has not yet been addressed for neuroactive pharmaceuticals. Moreover, according to the read-across hypothesis, for a given pharmaceutical, if the human target is conserved in fish, a pharmacological or ultimately toxicological effect is expected if concentrations reach human therapeutic concentrations (Huggett et al., 2003). However, the relation between internalized concentrations and fish biological responses is also not fully understood for most pharmaceuticals, though being critical for establishing the link between pharmaceutical exposure and toxicity effects (Miller et al., 2018; Rand-Weaver et al., 2013).

Aims and importance of this thesis

The main goal of this thesis is to study the presence of pharmaceuticals in the estuarine environment and investigate the effects of exposure in non-target fish species. Specifically, the present thesis aims to provide an overview of the limited knowledge available on the topic of neuroactive pharmaceuticals as well as provide key new insights on potentially threatening compounds by exploring their presence in the aquatic environment and their impacts in marine and estuarine fish species.

In this context, an overview of the scientific advances on the topic is given through a critical review of the current literature, where an innovative and integrated analysis of published data reveals important aspects of the ecotoxicology of neuroactive pharmaceuticals of human and veterinary use in fish species. Here, the use of lipophilicity as a predictor of uptake and bioconcentration of neuroactive pharmaceuticals is investigated, revealing a weak relation which in turn hinges on a multitude of experimental factors such as species, life-stage, tissue, among others. Results are discussed towards the classification of major hazard pharmaceuticals in the context of environmental risk assessments.

Aiming at investigating pharmaceuticals occurrence and spatial variability in the estuarine environment, the occurrence and bioaccumulation of neuroactive pharmaceuticals across seven fish species with distinct life-strategies is assessed, providing innovative and crucial insights. Overall, an innovative integrated framework is presented, providing an overview of the occurrence patterns across multiple estuarine systems, multiple species, multiple tissues and multiple pharmaceuticals.

A comprehensive approach of pharmaceuticals' ecotoxicology, through acute and chronic exposures of fish to pharmaceuticals, is also provided, aiming at evaluating effects at different levels of biological organisation (i.e. sub-individual and individual-level responses) and estimating inherent exposure risk. Two ecotoxicity studies were conducted in two different fish species, one estuarine resident and one marine species, in the short and long term, where a multi-biomarker approach is used for measuring changes from essential molecular/biochemical processes, up to individual key processes such as growth, condition and behavioural endpoints.

Overall, although pharmaceutical compounds have long been present in estuarine and marine environments, only recently are we awakening to their potential detrimental impacts and realising key information on the effects and the environmental risk they pose is still limited, particularly in the estuarine and marine environments. Hence, this thesis intends to expand the knowledge on the occurrence and effects of pharmaceuticals with estuarine field assessments

to determine neuroactive pharmaceuticals occurrence and variability patterns. Moreover, it intends to assess effects of exposure to non-target species through experimental trials, integrating multi-biomarker responses at sub-individual and individual levels in different fish species, ultimately contributing towards a more comprehensive and effective risk assessment of the impacts of these compounds in estuarine environments. Ultimately, the outcomes of this thesis aim to contribute with new insights of potential application in regulatory frameworks on pharmaceuticals by increasing recognition of the environmental occurrence and risk of exposure to neuroactive pharmaceuticals, and improve risk management policies within presently implemented environmental quality directives (e.g. Water Framework Directive, European Commission, 2000).

Thesis outline

The present thesis is composed of four scientific papers published in peer-reviewed international journals, each corresponding to a chapter.

In Chapter 2, a review of the current literature concerning bioconcentration and toxic effects following exposure of non-target fish species to neuroactive pharmaceuticals is presented. Here, by exploring available toxicity data, the relation between bioconcentration of neuroactive pharmaceuticals and their lipophilicity is studied, major drivers of bioconcentration are explored and the link between internalized concentrations and toxic effects concerning fish survival, growth and condition, behaviour and reproductive endpoints is determined. Ultimately, the comparison of toxic concentrations with current environmental concentrations enables the identification of major exposure risks and identify potentially critical pharmaceutical compounds in these natural ecosystems.

In Chapter 3, the occurrence and bioaccumulation of 33 neuroactive pharmaceuticals in surface waters and seven estuarine and marine fish species of four differently impacted estuarine systems are investigated. Here, the presence of neuroactive pharmaceuticals from seven different classes, such as antidepressants, antiepileptics, psychostimulants, opioids, or anxiolytics, in estuarine waters and their bioaccumulation patterns in various fish tissues, including brain, liver and muscle, are explored. Moreover, the links between bioaccumulation and compounds' lipophilicity, species habitat use patterns and trophic levels are also explored and discussed within the context of future environmental risk assessments.

In Chapter 4, the biological effects of short-term exposure to antidepressant fluoxetine on a resident estuarine fish species are explored. Multi-biomarker responses at sub-individual and individual levels are determined, including alterations to antioxidant and detoxification

mechanisms, cellular damage and neurotoxicity, as well as changes to individual responses such as activity and feeding behaviours.

In Chapter 5, the toxicity of long-term exposure to three pharmaceuticals with distinct modes of action and from different therapeutic classes (the antidepressant fluoxetine, the anti-hypertensive propranolol and the non-steroidal anti-inflammatory drug diclofenac) are investigated. Here, juveniles of a marine fish species are exposed to identify major impacts at the sub-individual and individual levels, including changes in antioxidant and detoxification processes, energy metabolism, neurotransmission and oxidative damage, as well as fish growth rate and pharmaceutical uptake.

Finally, a general discussion is presented in Chapter 6, where an integrated view of the main outcomes is presented, and future perspectives and knowledge gaps for further investigation are pointed out.

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CHAPTER 2

Bioconcentration of neuroactive pharmaceuticals in fish: Relation to lipophilicity, experimental design and toxicity in the aquatic environment

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Bioconcentration of neuroactive pharmaceuticals in fish: Relation to lipophilicity, experimental design and toxicity in the aquatic environment

Literature review	Bioconcentration	Environmental risk
▪ Neuroactive pharmaceuticals ▪ Bioconcentration ▪ Exposure effects ▪ Fish	▪ Relation with lipophilicity ▪ Influence by experimental design ▪ Link to toxicity (survival, growth, condition, reproduction, behaviour)	▪ Current environmental concentrations ▪ Freshwater, brackish and marine ecosystems

Abstract

Uptake of contaminants is linked to their toxicity and is usually estimated through their lipophilicity ($logK_{ow}$). Here, we review current literature regarding bioconcentration, i.e. uptake of contaminants from the external environment only, and the effects of exposure to neuroactive pharmaceuticals in fish. We aim to determine if lipophilicity is a suitable predictor of bioconcentration of these compounds in fish, to identify major drivers of bioconcentration and explore the link between bioconcentration potential and toxicity, focusing on survival, growth, condition, behaviour and reproduction endpoints. Additionally, we compare concentrations known to elicit significant effects in fish with current environmental concentrations, identifying exposure risk in ecosystems. The majority of studies have focused on antidepressants, mainly fluoxetine, and encompasses mostly freshwater species. Few studies determined pharmaceuticals bioconcentration, and even a smaller portion combined bioconcentration with other toxicity endpoints. Results show that lipophilicity isn't a good predictor of neuroactive pharmaceuticals' bioconcentration in fish, which in turn is highly influenced by experimental parameters, including abiotic conditions, species and life-stage. The need for increased standardization of experimental settings is key towards improving accuracy of environmental risk assessments and application in future regulatory schemes. Still, increased fish lethality was linked to increased bioconcentration, yet no other correlations were observed when considering effects on growth, condition, behaviour or reproduction, likely as a result of insufficient and variable data. In the

context of current environmental concentrations, several neuroactive pharmaceuticals were found to be potentially threatening, while data on occurrence is lacking for some compounds, particularly in brackish/marine systems. Specifically, nine compounds (fluoxetine, citalopram, sertraline, amitriptyline, venlafaxine, clozapine, carbamazepine, metamfetamine and oxazepam) were found at concentrations either above or critically close to minimum response concentrations, thus likely to affect fish in freshwater and brackish or marine environments, which supports further exploration in risk management strategies and monitoring programs in aquatic environments.

Keywords

Literature review, Psychoactive compounds, Uptake; BCF, Toxicology, Environmental risk

Introduction

The input of pharmaceuticals of both human and veterinary use into the aquatic environment results mainly from the direct release of municipal, hospital and industrial wastewaters, as well as aquaculture and animal husbandry discharges (Arnold et al., 2014; Cunha et al., 2017; Kümmerer, 2009; Tang et al., 2020). This constant release contributes to their pervasive and persistent presence, currently accounting for more than 600 pharmaceutical residues detected in aquatic ecosystems worldwide (Arnold et al., 2014; aus der Beek et al., 2016). Although typically found in concentrations at high ng/L range (aus der Beek et al., 2016), in some cases, close to specific hot-spots, concentrations can reach hundreds of μg/L (e.g. Fick et al., 2009). As pharmaceutical compounds target specific pathways, many of them conserved among vertebrates (Gunnarsson et al., 2019, 2008), their presence in the aquatic environment raises concerns over potential toxic effects to non-target species. Accordingly, alterations to numerous biological endpoints such as development, reproduction or behaviour have been reported mainly over the last two decades, for pharmaceuticals of various therapeutic classes, many at environmentally relevant concentrations (e.g. Corcoran et al., 2010; Fabbri, 2015; Fent et al., 2006; Overturf et al., 2015). In this context, several authors have highlighted the problematics of environmental contamination by pharmaceuticals (e.g. Daughton and Ternes, 2001; Fent et al., 2006; Halling-Sørensen et al., 1998), contributing to its classification as of emerging environmental concern, potentially threatening the aquatic environment and human health (European Parliament and Council, 2013).

Of particular relevance are neuroactive pharmaceuticals that act directly in the nervous system and, by interfering with chemical signalling associated with brain function, may

compromise essential physiological processes, ultimately leading to population-level effects through behavioural and reproductive changes (Brodin et al., 2014; Calisto and Esteves, 2009; Rang et al., 2012). A growing bulk of literature has focussed on these compounds, with an increasing number of studies measuring their environmental occurrence, bioconcentration and numerous biological effects following neuroactive pharmaceuticals exposure, albeit in a non-systematic manner and with varying degree of effects reported (e.g. Calisto and Esteves, 2009; Cunha et al., 2019, 2017; Sehonova et al., 2018). Among many other, changes to fish growth, reproduction and behaviour are pointed as of higher ecological relevance and potentially threatening for fish populations (e.g. Brodin et al., 2014; Hamilton et al., 2016; Melvin and Wilson, 2013).

Bioconcentration, on the other hand, is also frequently considered, in line with potential increased toxicity and transference within food webs, and it occurs in various tissues, such as brain, muscle, plasma or liver (e.g. Duarte et al., 2020; Heynen et al., 2016; Xie et al., 2017). Bioconcentration is inherently associated with compounds' physical and chemical properties, particularly with lipophilicity. Estimated through octanol/water partition coefficient (K_{ow}), lipophilicity describes the partition of compounds between polar (water) and non-polar (octanol) fractions. Octanol is considered a surrogate for lipid-rich organism tis-sues/membranes, and thus used as a proxy for bioconcentration of lipophilic compounds. Accordingly, there is a general agreement that bioconcentration of lipophilic compounds increases as $\log K_{ow}$ increases, for compounds with $\log K_{ow}$ up to 6 (e.g. Arnot and Gobas, 2006; Bintein et al., 1993; Mackay, 1982). Since the early 2000s, the European Chemicals Agency (ECHA) and the European Medicines Agency (EMA) guidelines set lipophilicity level as a threshold ($\log K_{ow} > 3$ or 4.5) as a requirement for further evaluation of bioconcentration and bioaccumulation potential as well as for risk assessment analysis of newly designed chemicals and pharmaceuticals, respectively.

In the specific case of pharmaceuticals, the increased awareness of potential toxicity to non-target organisms contributed to its current classification as compounds of emerging environmental concern and recent inclusion in European legislation (European Parliament and Council, 2013). However, the presence of pharmaceuticals in the environment is still largely unregulated, mostly due to a lack of risk assessment data for compounds that entered the market before any legislation was applicable (Kuster and Adler, 2014). Moreover, the link between higher bioconcentration potential and increased toxicity in fish has not yet been explored for neuroactive compounds, though it is generally accepted for organic compounds (e.g. Sijm and Hermens, 2005).

In this context, this study aims to provide new insights regarding bioconcentration and biological effects of exposure in fish, based on currently available literature. Specifically, it aims to determine if bioconcentration of neuroactive pharmaceuticals i) can be predicted by compounds' lipophilicity, ii) is influenced by experimental conditions namely pH, temperature, salinity and exposure time, iii) is associated with increased toxicity to fish, affecting survival, growth, condition, behaviour or reproduction. Ultimately, we compare current environmental neuroactive pharmaceutical levels with concentrations known to elicit significant effects, highlighting imminent risks of exposure in natural freshwater as well as brackish and marine ecosystems.

Materials and Methods

Literature search and inclusion criteria

Publications used in this study were compiled following a systematic search in Web of Science (WoS, 28/08/2020), of unlimited time range, which targeted studies exclusively on fish and neuroactive pharmaceuticals, with keywords including "Fish", "Pharmaceutic*" and different combinations of terms used to define pharmaceutical classes and endpoints measured. For pharmaceutical classes, terms of the Anatomical Therapeutical Chemical (ATC) classification system from WHOCC (see Appendix 1, Table A1.1) were considered for all classes within category N (which includes all compounds acting on the nervous system), along with other common terms used, such as neuroactive, psychoactive or psychiatric. Furthermore, multiple keywords were selected to target potential endpoints measured such as growth, behaviour, reproduction, bioconcentration, among others (for full details on the research steps and terms used see Table 2.1 and Appendix 1, Fig. A1.1).

Table 2.1. Research terms used for systematic literature search.

Animal group	Fish
Response/Endpoint	respons* OR effect* OR expos* OR assay* OR toxic* OR growth OR morphometric* OR condition OR behavio* OR reproduct* OR bioconcen* OR uptake OR bioaccum* OR LC50 OR EC50 OR NOEC OR LOEC
Compound	Pharmaceutic* OR

ATC class (1st and 2nd levels) and other common terms	analgesic* OR painkill* OR antipyretic* OR opioid* OR antimigraine
	anesthetic*
	antiepileptic* OR anticonvulsant*
	anti-parkinson
	psychoanaleptic* OR anti-dementia OR antidepressant* OR psychostimulant*
	psycholeptic* OR antipsychotic* OR hypnotic* OR sedative*
	anxiolytic*
	neuroactive
	psychoactive
	psychiatric

A database with more than 3500 distinct publications was created. A thorough selection of publications of interest was conducted, selecting studies that matched all the following criteria:

i. waterborne *in-vivo*, single generation exposure;
ii. single compound exposure;
iii. exposure within a controlled environment (excluding e.g. mesocosms, natural ponds);
iv. exposure without any prior or subsequent treatment/potential stressor (excluding e.g. pharmaceutical exposure followed by simulated transportation).

An additional search was also performed on references from review articles concerning neuroactive compounds, obtained from the systematic search, which resulted in the inclusion of 13 additional studies. A final database including 451 publications was obtained, with articles between 1979 and 2020 (see all references used in Appendix 1, Table A1.2).

Information concerning neuroactive compounds, concentrations, exposure time, species and tissues used, as well as abiotic parameters measured (temperature, pH, salinity) was included in the database. Additionally, for each endpoint, the Minimum Response Concentration (MRC) was registered, which corresponds to the lowest concentration at which a significant effect was observed. Endpoints considered for further analysis included:

i. Lethal concentration 50 (LC50);
ii. Growth and condition (growth rate, body length and weight, condition factor);

iii. Behaviour, including predatory avoidance and feeding behaviours (freezing/active time after predatory attack, latency to enter strike zone/leave refuge, number of entries/fish entering the strike zone, latency to reach cover/refuge, escape rate/velocity; number/frequency of feeding strikes, number of feeding individuals, time to first feeding, latency to capture i^{th} prey, number prey consumed/capture success, time to total intake, feeding rate);

iv. Reproduction, including hatching and fecundity endpoints (total number of eggs, eggs per female or day, clutch size, number of clutches per tank, spawning rate, time to initiate breeding; hatching success, number of eggs hatched, time to hatch).

Bioconcentration factors (BCF) were also included in the database, considering both reported values and values calculated as the ratio between measured tissue and water pharmaceutical concentrations, whenever sufficient information was provided. Bioaccumulation and kinetic derived factors were not included due to their distinctive nature and exceptionally low number.

Pharmaceuticals' ATC classification was obtained through the ATC search engine for pharmaceuticals of human (https://www.whocc.no/atc_ddd_index/) or veterinary use (https://www.whocc.no/atcvet/atcvet_index/). Pharmaceuticals were considered of veterinary use when found exclusively on veterinary ATC index, otherwise were considered of human use.

Pharmaceuticals' estimated log octanol-water partition coefficient values (log K_{ow}) for uncharged molecules, were obtained via KOWWIN™ program by EPI (Estimation Programs Interface) Suite™ (https://www.epa.gov/tsca-screening-tools/epi-suitetm-estimation-program-interface).

To link current environmental concentrations to concentrations known to trigger effects in fish, a comparison between environmental concentrations and the MRC of all endpoints considered was performed. Choice of MRC values followed the European guidelines on environmental risk assessment of chemical substances to human health and the environment (European Commission, 2003), which proposes a more conservative approach, that whenever information on both freshwater and brackish/marine species is available, the data should be pooled and the assessment should be based on the most sensitive endpoint, regardless of the medium. Back in 2016, aus der Beek and colleagues compiled worldwide measurements of pharmaceuticals in the environment, available in the literature until 2013. To complement their database with the most recently published research, we searched for environmental concentrations measured in

surface waters from 2013 to 2021, using environment/water and detection/quantification/screening as keywords in Web of Science (24/03/2021). Of all studies found, the average and maximum values reported for each pharmaceutical in freshwater and brackish/marine environments (exclusively on surface water samples, excluding any potential overestimation linked to pharmaceutical production sources or wastewater treatment effluents which are known to have exceptional contributions) were compiled (see Appendix 1, Table A1.3). A median value (of all average values compiled) was then calculated for each pharmaceutical and considered for graphical representation, along with maximum reported values.

Statistical analysis

Spearman rank correlation analysis was used to test for correlations between neuroactive pharmaceuticals' lipophilicity ($\log K_{ow}$) and bioconcentration factor (median BCF), as well as between median BCF and minimum response concentrations (MRC), for each endpoint class. Endpoints considered in the analysis included lethality (LC50), growth and condition, behaviour (predatory avoidance and feeding) and reproduction (hatching and fecundity).

Principal components analysis (PCA) was performed to explore the potential influence of abiotic parameters (pH, salinity and temperature) and exposure time on neuroactive pharmaceuticals' bioconcentration. Only BCF values for which all parameters were provided (pH, salinity, exposure time and temperature) were included in the analysis, i.e., a total of 218 out of 381 BCFs (see Appendix 1, Table A1.4).

All analyses were performed in R software (R Core Team, 2019), considering a significance level of 0.05.

Results

Literature on neuroactive pharmaceuticals

Over the last thirty years, an exponential increase in the number of publications concerning the effects of neuroactive pharmaceuticals in fish is evident (Fig. 2.1). Overall, less than 10 studies were published per year until 2006, whereas in the last decade a steady increase of publication rate followed, reaching up to 65 publications in the year of 2019. Until the 28th of August 2020, when our search was conducted, 31 studies had already been published. A total of 87 different neuroactive pharmaceuticals were studied among the 451 publications considered (Appendix 1, Table A1.2).

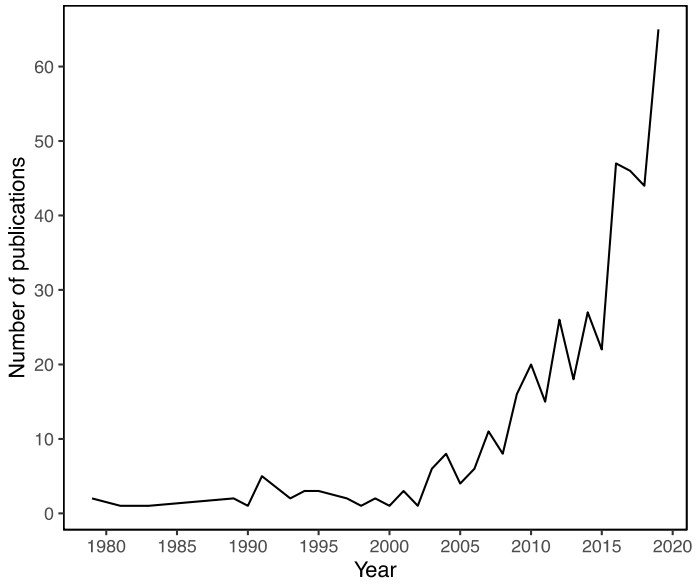

Figure 2.1. Rate of publications concerning bioconcentration and effects of neuroactive pharmaceutical compounds in fish, until 2019 (N=420).

Neuroactive compounds of human use accounted for 90.8% of all compounds studied, whilst veterinary use corresponded to 6.9%, and 2.3% were not listed in the ATC system. The most represented pharmaceutical classes were Psychoanaleptics (N06) and Psycholeptics (N05) corresponding to 32.2% and 23% of all compounds, respectively. Within the Psychoanaleptics class, the vast majority, 71.4%, were Antidepressants (N06A), whereas 21.4% corresponded to Psychostimulants, agents used for ADHD and nootropics class (N06B), and 7.1% to Anti-dementia drugs (N06D). Concerning Psycholeptic compounds, 45% were Antipsychotics (N05A), 35% Anxiolytics (N05B) and 20% Hypnotics and sedatives (N05C).

The most investigated pharmaceuticals were the antidepressant fluoxetine (98 publications), anesthetics tricaine mesylate (90) and benzocaine (49), the antiepileptic carbamazepine (41), antidepressants venlafaxine (22) and sertraline (21) and the anxiolytic diazepam (21).

Molecular changes (measured in 42.8% of all publications), alterations in fish behaviour (37.3%) and physiology and growth (27.7%) were the most frequently assessed endpoints, followed by mortality (26.6%) and anesthetic efficacy (23.9%). Pharmaceutical bioconcentration, accumulation or depuration in fish tissues was measured in 14% of all publications, whereas effects on fish reproduction or development and histology were investigated in only 12.9% and 10.4% of studies, respectively. Only 10.4% of all studies determined pharmaceutical uptake, concentration or depuration simultaneously to any other endpoints considered.

Among all publications, 125 different species and subspecies were investigated, yet almost half of all studies (47.9%) were restricted to four freshwater species: *Danio rerio*, *Oncorhynchus mykiss*, *Pimephales promelas* and *Carassius auratus*. Moreover, studies were

performed within freshwater (86%), brackish and marine (11.3%), both (1.3%) or undefined (1.3%) environmental conditions.

Bioconcentration and lipophilicity of neuroactive pharmaceuticals

A total of 381 bioconcentration factors (BCF) were available for 22 out of the 87 pharmaceutical compounds considered (Fig. 2.2). The majority of BCF values were measured in juveniles (46%) and adults (26%), followed by larvae (9%), embryos (7%) and 12% with undefined stage. The tissue groups used for BCF determination were the brain (17%), whole individual (18%), plasma (10%) and all the remaining organs (55%), which include muscle, liver, bile, gills, intestine, gonad, kidney and eyes. In terms of exposure time, 19% of studies determined BCF values following exposure up to 24 h or less, 52% within 1 to 7 days and 28% between 7 and 42 days.

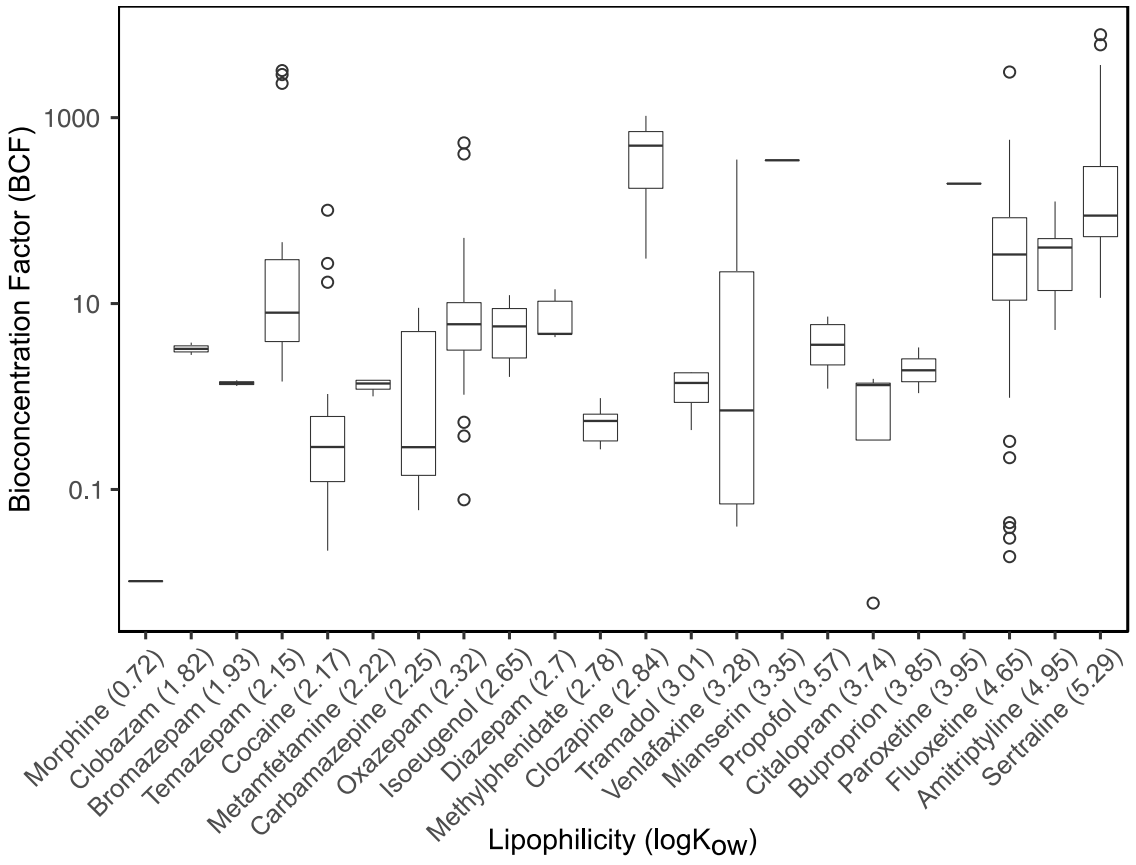

Figure 2.2. Bioconcentration factors (BCF, N=381) of neuroactive pharmaceuticals with increasing lipophilicity (logK_{ow}). Boxplots show median, 25th and 75th percentiles; upper and lower whiskers represent 1.5 times the interquartile range (IQR) of maximum and minimum values, respectively.

Spearman rank correlation tests revealed that bioconcentration factor was positively correlated to pharmaceutical lipophilicity ($r_s = 0.50$, p-value = 0.018), when considering median

values of all 22 pharmaceuticals (Fig. 2.2). For the two most represented groups, Psycholeptics and Antidepressants, no significant correlations between median BCF and lipophilicity were observed (r_s = 0.71, p-value = 0.136 and r_s = 0.36, p-value = 0.389, respectively; Fig. 2.3A and B).

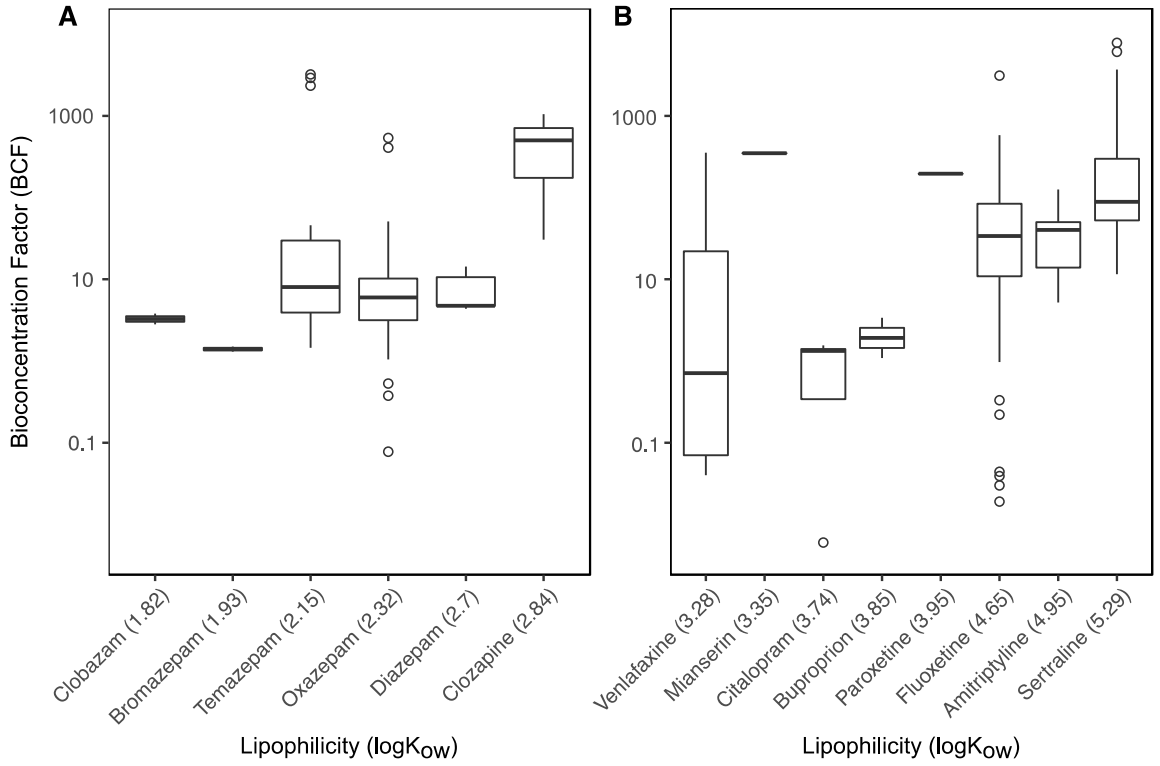

Figure 2.3. Bioconcentration factors (BCF) of Psycholeptics (A, N=88) and Antidepressants (B, N=205) groups with increasing lipophilicity ($logK_{ow}$). Boxplots show median, 25th and 75th percentiles; upper and lower whiskers represent 1.5 times the interquartile range (IQR) of maximum and minimum values, respectively.

Considering different tissue groups, a significant positive correlation was found between BCF and $logK_{ow}$ for whole individuals (r_s = 0.86, p-value = 0.024, Fig. 2.4A-D), which seems to be mainly driven by two compounds, cocaine and fluoxetine (Fig. 2.4A). Contrarily, non-significant correlations were observed for brain (r_s = 0.28, p-value = 0.325, Fig. 2.4B), plasma (r_s = 0.19, p-value = 0.608, Fig. 2.4C) and other organs group (r_s = 0.35, p-value = 0.215, Fig. 2.4D), which include muscle, liver, bile, gills, intestine, gonad, kidney and eyes.

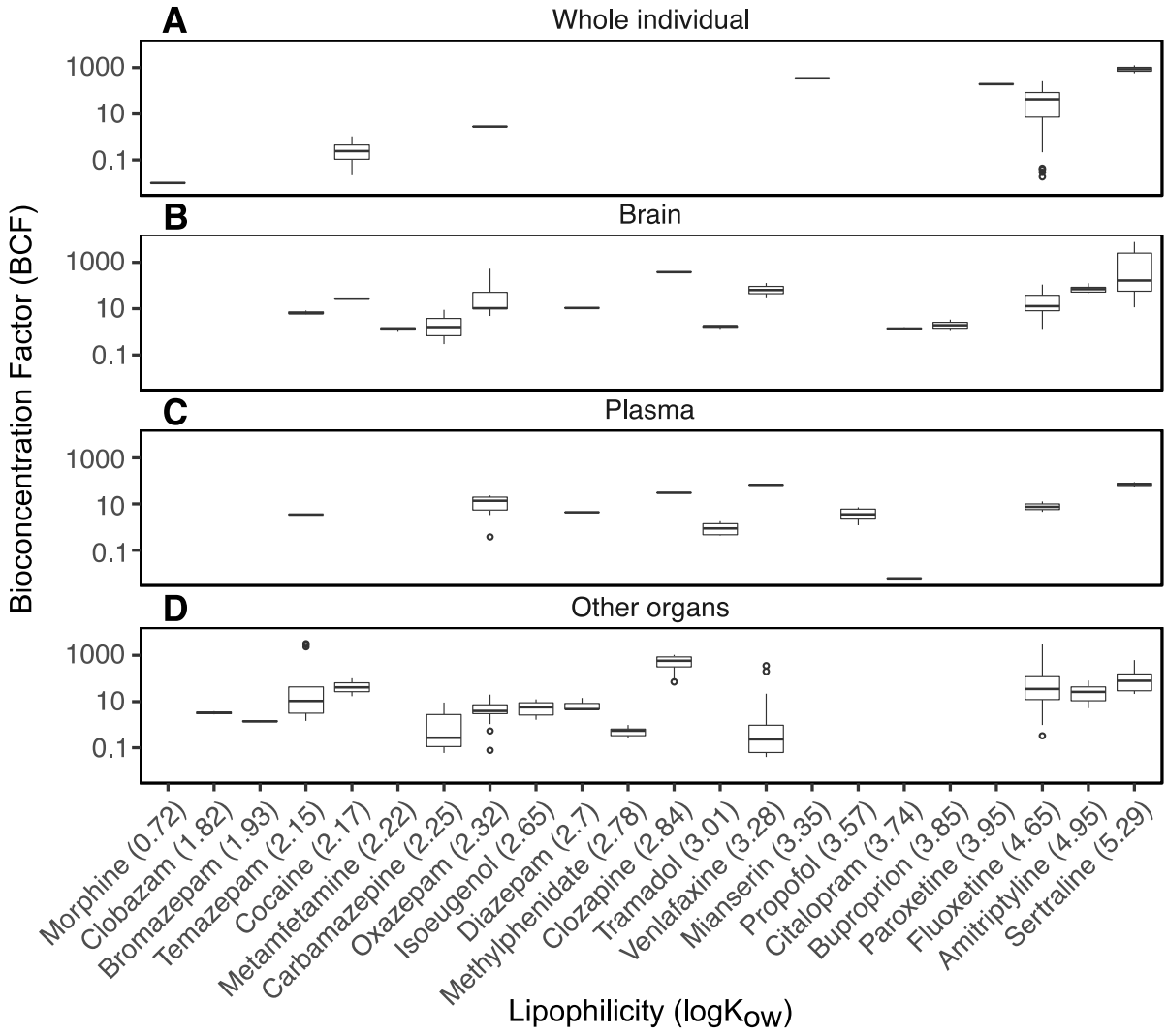

Figure 2.4. Bioconcentration factors (BCF, N=381) of neuroactive pharmaceuticals with increasing lipophilicity ($logK_{ow}$) in different tissue groups: (A) whole individual, (B) brain, (C) plasma and (D) other organs, including muscle, liver, bile, gills, intestine, gonad, kidney and eyes. Boxplots show median, 25th and 75th percentiles; upper and lower whiskers represent 1.5 times the interquartile range (IQR) of maximum and minimum values, respectively.

Bioconcentration variation with exposure conditions

Principal component analysis revealed that 67.29% of BCF variation can be explained by the first two principal components. Overall, the analysis evidenced patterns of data distribution driven mostly by temperature and to a lesser extent by exposure time (Fig. 2.5). Pharmaceuticals with high bioconcentration potential, corresponding to BCF quartiles Q3 and Q4, are mostly represented at the lower-right side of the plot, closely associated with variations in temperature and exposure time, although a few are positioned at the centre of the diagram. Most of these compounds correspond to antidepressants (N06A), but also hypnotics and sedatives (N05C) and antipsychotics (N05A). On the central and upper part of the graph are compounds with low BCFs (Q1 and Q2), particularly anxiolytics (N05B), psychostimulants (N06B) and

antiepileptic (N03A) pharmaceuticals, where differences in temperature contribute to their separation into two clusters.

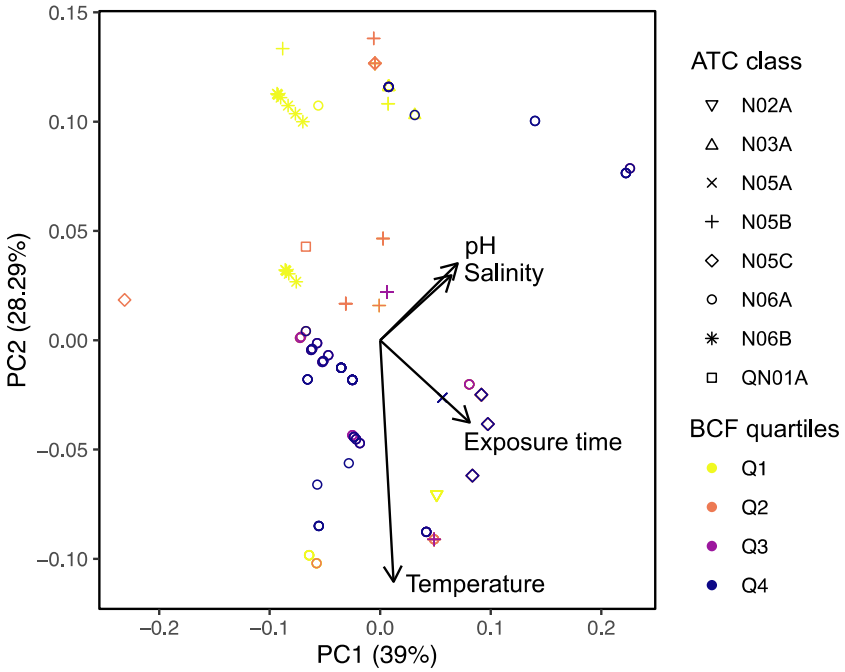

Figure 2.5. Principal component analysis (PCA) of bioconcentration factors (BCF) and experimental conditions, namely pH, salinity, temperature and exposure time. Data points are coloured according to BCF quartiles (increasing BCF values from Q1 to Q4) and different shapes correspond to different ATC classes, namely opioids (N02A), antiepileptics (N03A), antipsychotics (N05A), anxiolytics (N05B), hypnotics and sedatives (N05C), antidepressants (N06A), psychostimulants, agents used for ADHD and nootropics (N06B) and veterinary anesthetics (QN01A).

Bioconcentration and toxicity

Neuroactive pharmaceuticals' bioconcentration was negatively correlated with LC50 (r_s = -0.76, p-value = 0.016; Fig. 2.6A), meaning that neuroactive compounds with increasing bioconcentration are more lethal to fish (have lower LC50). However, no significant correlations were observed between pharmaceuticals' BCF and any other endpoint considered, specifically: growth and condition (r_s = 0.30, p-value = 0.431, Fig. 2.6B), behaviour (r_s = -0.05, p-value = 0.912, Fig. 2.6C) or reproduction (r_s = -0.33, p-value = 0.359, Fig. 2.6D).

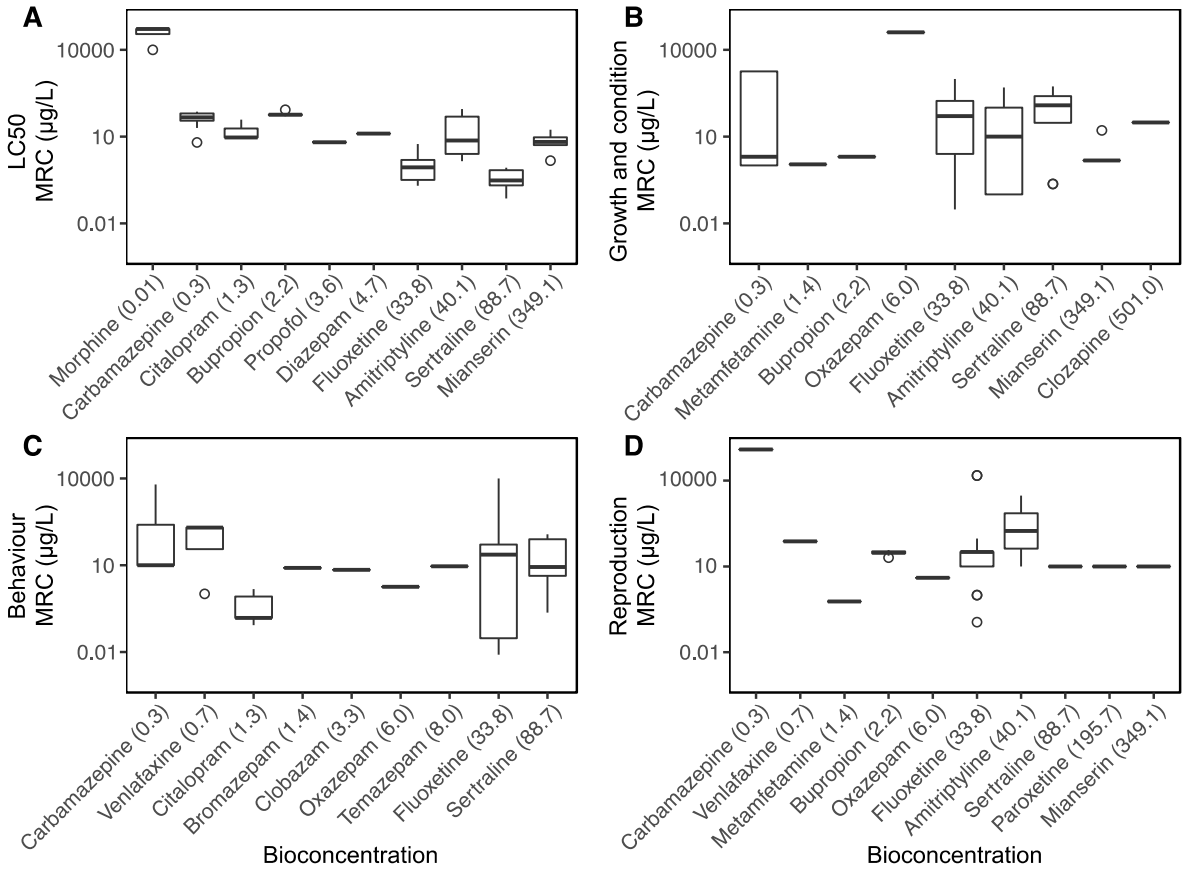

Figure 2.6. Minimum response concentrations (MRC, µg/L) of neuroactive pharmaceuticals with increasing median bioconcentration factor: (A) Lethality (LC50), (B) Growth and condition, (C) Behaviour (predatory avoidance and feeding) and (D) Reproduction (fecundity and hatching). Boxplots show median, 25th and 75th percentiles; upper and lower whiskers represent 1.5 times the interquartile range (IQR) of maximum and minimum values, respectively.

Biological effects and environmental concentrations

Minimum response concentrations were available for 18 compounds of different therapeutic classes (Fig. 2.7 and Appendix 1, Table A1.5). All endpoint classes considered were represented, with alterations to behaviour described for many compounds (8 out of 18), followed by effects on growth and condition (6) and reproduction (2). For anxiolytic diazepam, opioid morphine and anesthetic propofol no significant effects were reported within these endpoint classes, therefore the MRC values presented correspond to lethal effects (LC50).

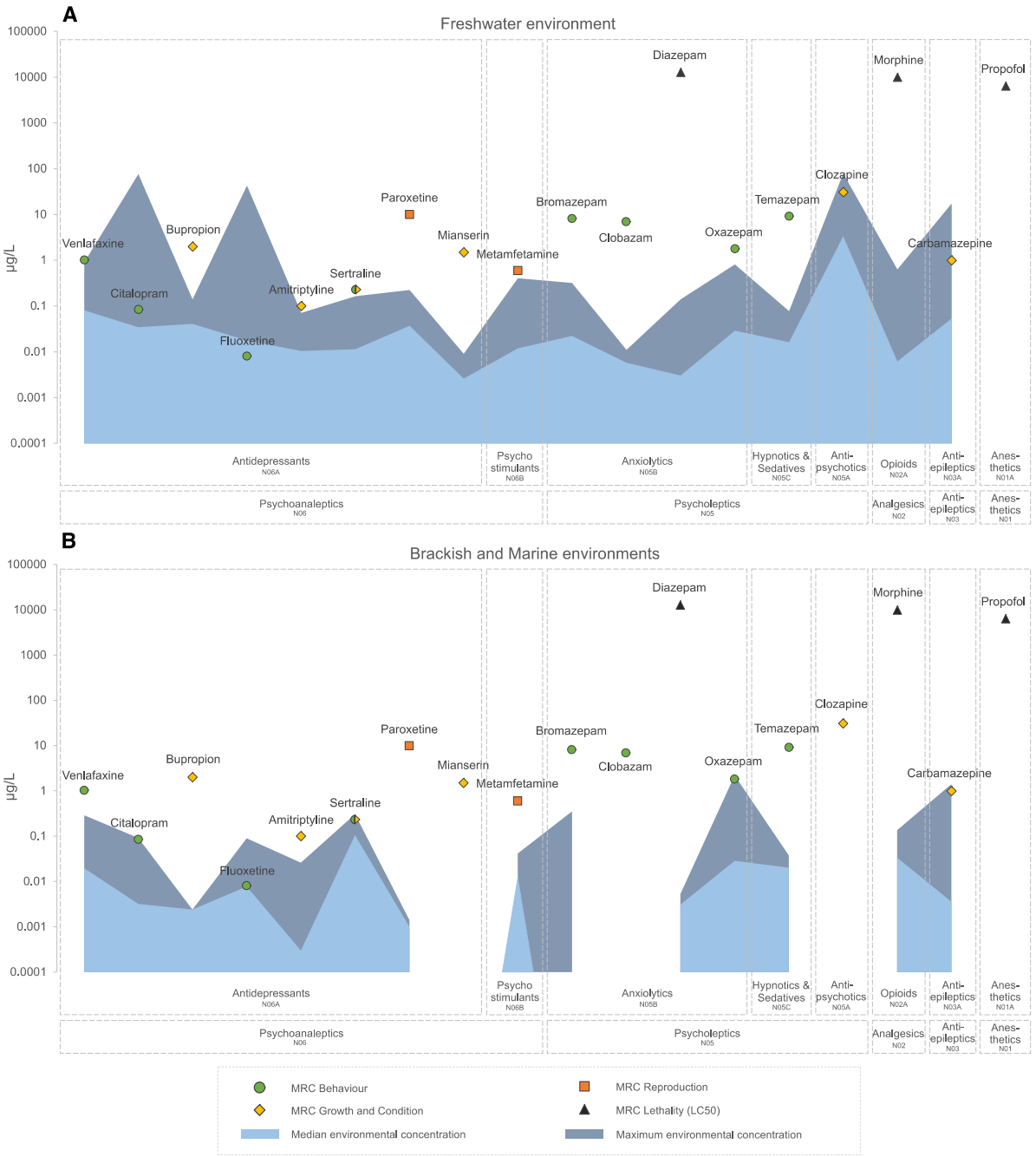

Figure 2.7. Minimum response concentrations (MRC, µg/L), median and maximum environmental concentrations (µg/L; light and dark blue areas, respectively) of 18 neuroactive pharmaceuticals from different classes in (A) freshwater and (B) brackish and marine environments. MRC values correspond to the lowest concentration at which a significant effect was described in fish. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Current environmental concentrations of pharmaceuticals in surface waters have been reported in the range of ng/L to µg/L. Measurements were more frequent in freshwater (71.3%) than in brackish or marine environments (28.8%), and concentrations tended to be higher in freshwater systems. The highest concentrations detected in freshwater were 78.3 µg/L of clozapine and 76 µg/L of citalopram, whereas in brackish and marine systems the highest values reported were 2.2 µg/L of oxazepam and 1.4 µg/L of carbamazepine. To the best of our

knowledge, propofol has not yet been detected in surface waters and clobazam, clozapine and mianserin have been found exclusively in freshwater environments.

Within freshwater systems (Fig. 2.7A), median concentrations surpass fluoxetine MRC by over 2-fold, and maximum reported concentrations over 5000-fold. Likewise, surface water concentrations of antiepileptic carbamazepine represent a significant risk to fish growth and condition, with maximum concentrations exceeding the MRC by 17 times. Maximum reported concentrations of antipsychotic clozapine exceed MRC for fish growth and condition by 2.5 times, whereas median concentrations are ten times lower than MRC. With environmental concentrations practically reaching MRCs of antidepressants venlafaxine, amitriptyline and sertraline, changes in fish behaviour and growth and condition are imminent, as well as of psychostimulant metamfetamine (commonly referred to as methamphetamine), likely to cause reproductive changes in fish.

In brackish and marine environments (Fig. 2.7B), antidepressant fluoxetine median environmental concentration equals MRC for behaviour whereas maximum concentrations exceed MRC by 11 times. Likewise, MRCs for antidepressants citalopram and sertraline, anxiolytic oxazepam and antiepileptic carbamazepine are exceeded by maximum surface water concentrations. Notably, following the most sensitive data criterion (European Commission, 2003), only one MRC value corresponded to a study done in brackish/marine conditions (Diazepam, Figure 2.7B, see also Appendix 1, Table 1.5).

Data on the occurrence of neuroactive compounds was only available for 14 out of 18 compounds for brackish and marine environments, in contrast with freshwater systems, where data was available for 17 out of 18 compounds. Nonetheless, for three compounds, namely sertraline, bromazepam and oxazepam, maximum concentrations detected in brackish or marine environments exceed those reported for freshwater systems.

Discussion

Literature on neuroactive pharmaceuticals

An increasing amount of literature has been published over the last decades, concerning the potential effects of pharmaceuticals on non-target organisms. In particular, studies of neuroactive compounds effects in fish are steadily increasing, with the publication rate thriving in the first years of 2000 and reaching up to 65 publications in 2019. Still, the exploration of the effects of different compounds in fish alone is unbalanced. Out of the 451 studies considered

in this review, encompassing 87 neuroactive compounds, more than half explored the effects of psycholeptics and psychoanaleptic drugs.

Fluoxetine was, by far, the most studied neuroactive pharmaceutical of human use, considered in 98 publications. Following evidence of serotonin modulation in fish by fluoxetine and first indications of adverse effects in fish (Brooks et al., 2003b, 2003a), an increased effort to further assess fluoxetine and other antidepressants exposure effects was evident (e.g. Brooks, 2014; Sehonova et al., 2018). Notably, selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine, sertraline and citalopram, as well as antidepressants from other classes (e.g. venlafaxine, amitriptyline, mianserin, bupropion) have been found to bioconcentrate in fish tissues and to cause significant alterations to behaviour, growth, condition or reproduction (e.g. Duarte et al., 2020; Hubená et al., 2020; Nowakowska et al., 2020; Painter et al., 2009; Sehonova et al., 2017). In this context, antidepressants venlafaxine and sertraline are also among the most studied compounds, with more than 20 studies each.

Veterinary anesthetic tricaine mesylate and anesthetic of human use benzocaine were also markedly explored (90 and 49 studies, respectively), yet essentially within the context of its common use in aquaculture, to reduce fish stress associated with transport and handling (e.g. Coyle, 2004). The effects of exposure to antiepileptic carbamazepine were also vastly addressed (in 41 publications), mostly encouraged by its wide distribution and frequent detection in the aquatic environment, as a result of its low biodegradability (aus der Beek et al., 2016; Zhang et al., 2008).

A substantial discrepancy between the number of studies with brackish or marine species and freshwater species is also evident. The vast majority of studies, i.e. 86%, were performed exclusively in freshwater conditions, whereas only 11.3% of studies were performed within brackish or marine conditions. This divergence has previously been reported by several authors (Fabbri and Franzellitti, 2016; Fonseca and Reis-Santos, 2019; Gaw et al., 2014), emphasizing the need for further assessment of occurrence and effects in brackish and marine environments, primarily overlooked due to presumed dilution and dispersion processes in these ecosystems. Notwithstanding, multiple sources of pharmaceutical input to marine and coastal areas prevail, including direct wastewater discharges decidedly contributing to increased pharmaceutical input (Gaw et al., 2014). Moreover, despite more than 120 species and subspecies being targeted in all studies, almost half of all studies (47.9%) were restricted to four freshwater species: *Danio rerio*, *Oncorhynchus mykiss*, *Pimephales promelas* and *Carassius auratus*. Importantly, intra and interspecies differences have been documented (e.g. Villeneuve et al., 2010;

Vossen et al., 2020), and therefore, the use of different species is warrant to account for natural variability and potentially dissimilar susceptibilities.

Of all endpoints considered, effects on fish physiology and growth, behavioural, molecular changes and mortality were the most frequently measured endpoints. Uptake, accumulation or depuration were considered by only a small portion of studies (14%), and in only one tenth of those it was assessed in combination with any other endpoints, although the combination of both bioaccumulation and ecotoxicity has been considered a promising approach to environmental risk assessment for many years (Franke, 1996; van der Oost et al., 2003). Reproductive effects were the least frequently assessed endpoints, probably associated with the lower likelihood of such effects occurring (Lopes et al., 2020) and the longer exposure periods usually required to assess such endpoints in fish (Melvin and Wilson, 2013).

Data scarcity was also evident for bioconcentration factors (BCF), only covering 22 out of the 87 compounds listed. For three compounds, namely morphine, mianserin and paroxetine, only single BCF values were available, and for ten other compounds bioconcentration factors were limited to single studies. Hence, while the estimation of bioconcentration factors is limited to only one fourth of all studied compounds, it is also, for more than half of the 22 compounds, largely limited to the specificity of individual studies, including particular species, life-stages or tissues.

Bioconcentration and lipophilicity of neuroactive pharmaceuticals

Hydrophobic compounds have an increased propensity to partition into the lipidic fraction, in this case, biota tissues (e.g. Arnot and Gobas, 2006). This is in fact a characteristic of interest, sought for in pharmaceutical production, along with low molecular weight, as it increases permeability across biological membranes, especially for compounds that are intended to cross the blood-brain barrier (Pardridge, 2007). Bioconcentration of lipophilic chemicals has been shown to increase with increasing lipophilicity levels, and many predictive models have been developed to describe this correlation (Arnot and Gobas, 2006; Bintein et al., 1993; Mackay, 1982). Accordingly, lipophilicity thresholds have been set and are used to predict chemical bioaccumulation and potential environmental risk in European legislation since 2006 (European Parliament and Council, 2006).

However, our results show a weak although significant correlation between lipophilicity and bioconcentration considering all 22 pharmaceuticals for which BCF data was available (47 studies). We also found no significant correlations between lipophilicity and bioconcentration within two most represented therapeutic groups, antidepressants and psycholeptics. Markedly,

some compounds with apparently no bioconcentration potential according to European guidelines (i.e., $logK_{ow}$ < 3/4.5), are shown to extensively bioconcentrate in fish. For instance, the antipsychotic clozapine, which would not be considered as of substantial bioconcentration potential ($logK_{ow}$ = 2.84), presented the highest median BCF of all compounds, and was shown to bioconcentrate in fish tissues 358 times more than tramadol ($logK_{ow}$ = 3.01), the first pharmaceutical to fall beyond the threshold of 3. Likewise, benzodiazepines temazepam and oxazepam ($logK_{ow}$ = 2.15 and 2.32, respectively) have higher median BCFs than some compounds with $logK_{ow}$ between 3.5 and 3.8, such as antidepressants citalopram and bupropion.

Considering different tissue groups, bioconcentration was only significantly correlated to lipophilicity for whole-individual measurements, and no correlation was observed for brain, plasma or other organs groups, which include muscle, liver, bile, gills, intestine, gonad, kidney and eyes. However, in the whole-individuals group, the correlation found was driven mainly by two compounds, cocaine and fluoxetine, and by single BCF values (morphine, oxazepam, mianserin and paroxetine), which results in extrapolation to all other neuroactive compounds.

Overall, lipophilicity should not be considered the best predictor for bioconcentration of neuroactive pharmaceuticals in fish. Previous studies have highlighted the apparent under or overestimation of pharmaceutical uptake with lipophilicity-based predictions (e.g. Boström et al., 2017; Fick et al., 2010). Moreover, the use of a cut-off value of 3 without any further toxicity assessment, has already been considered doubtful (Franke, 1996). Therefore, the adjustment of current chemical regulations of these compounds to include assessment of compounds with lipophilicity lower than the current thresholds is paramount. Instead, the approach should be based, whenever possible, on empirical bioconcentration factors. Particularly, the measurement of pharmaceutical concentrations in fish plasma allows for direct comparison with human therapeutic concentrations and is therefore convenient for predicting effects in fish. The application of such an approach has also previously been suggested to improve risk assessment (Ågerstrand et al., 2015; Huggett et al., 2003; Rand-Weaver et al., 2013). Notwithstanding, varying metabolism and drug specific pharmacokinetics, which are not accounted for in our results/data, can also influence the bioconcentration of these compounds. Recent information on drug pharmacokinetics and species metabolism reveals their fundamental role in bioconcentration, pointing to the need for considering these factors in future BCF determinations. For example, Margiotta-Casaluci et al., (2014) described a bi-phasic kinetics in fluoxetine concentration in plasma of fathead minnow as a result of increased fluoxetine metabolism at higher concentrations. Tanoue et al., (2017) discussed how clearance rates and plasma protein binding profiles might influence tramadol plasma concentrations in the same species, whereas Huerta et al., (2016) reported

different oxazepam bioconcentration profiles between fathead minnow males and females, probably linked to gender-specific traits.

Overall, by including such approaches in regulatory legislation, misclassification of newly produced chemicals, as well as of presently marketed drugs, based solely on lipophilicity, would certainly be avoided and contribute to improved environmental risk assessment.

Bioconcentration variability with exposure conditions

For many compounds, such as venlafaxine, oxazepam or fluoxetine, a substantial variability of BCFs was evident. Besides the inherent properties of pharmaceutical compounds, differences in bioconcentration among studies are likely to occur if different species, life-stage, exposure conditions, among other factors, are considered (Arnot and Gobas, 2006; Franke, 1996; Miller et al., 2018; Sijm and Hermens, 2005; van der Oost et al., 2003). Noteworthy, the principal component analysis revealed that more than 67% of BCF variability could be attributed to differences in experimental parameters, mainly to temperature but also exposure time, salinity and pH, highlighting the importance of such parameters in BCF assessment of neuroactive pharmaceuticals. The analysis also revealed the varying effects of these parameters within and among neuroactive pharmaceutical classes, revealing the complex nature of pharmaceuticals' bioconcentration. Only rarely the influence of such experimental parameters on neuroactive pharmaceutical uptake has been considered, yet some effects have been reported: Nakamura et al. (2008) and Alsop and Wilson (2019) showed highly pH-dependent fluoxetine and sertraline bioconcentration on body and liver of *Oryzias latipes* juveniles and in *Danio rerio* larvae, respectively; McCallum et al. (2019) and Meinertz et al. (2006) showed temperature influence on methylphenidate bioconcentration in *Pungitius pungitius* muscle and on isoeugenol uptake in *Oncorhynchus mykiss* muscle, respectively. The effect of exposure time is also rarely considered, with only a few studies presenting more than one sampling event. For instance, Xie et al. (2015) reported higher sertraline BCFs after 7 days when compared to 4 days exposure in *Carassius auratus* juveniles. Ziarrusta et al. (2017) also reported increasing BCFs with exposure time (2, 4 and 7 days), in *Sparus aurata* juveniles' tissues exposed to amitriptyline. In a study by Pan et al. (2018), higher fluoxetine BCF was observed after 6 days than after only 3 days both in *Danio rerio* adults and *Carassius auratus* juveniles. Overall, higher BCF values are obtained with increasing exposure time, which is likely related to the time taken to reach a steady-state (Arnot and Gobas, 2006; Sijm and Hermens, 2005). Notably, this increasing BCF over-time is more pronounced at lower concentrations and in fish brain, than in any other tissues such as muscle, liver or gills (e.g. Pan et al., 2018; Xie et al., 2015; Ziarrusta et al.,

2017), stressing the importance of concentration, exposure time and tissue selection in the assessment of BCF. In this context, the relevance of chronic toxicity studies is frequently acknowledged, contributing to more accurate predictions of environmental exposure compared to short-term toxicity events, given the persistent nature of pharmaceuticals in aquatic systems (Crane et al., 2006; Fent et al., 2006). However, only 28% of BCFs were determined within longer timeframes (more than 7 days), despite the fact that some neuroactive pharmaceuticals require chronic administration to achieve the desired pharmacological effects in humans (Kreke and Dietrich, 2008).

Concerning fish tissues, increased concentrations of pharmaceuticals in bile and liver are predictable, given their role in detoxification, whilst for neuroactive pharmaceuticals, increased bioconcentration in fish brain is also likely (Miller et al., 2018; van der Oost et al., 2003). Accordingly, bile presented particularly high bioconcentration factors (up to 2000x), despite sparse measurements, in comparison to other tissues, including liver, brain and muscle (Togunde et al., 2012; Zhao et al., 2017). In general, liver either showed increased or similar BCFs to fish brain, yet both tissues presented much higher bioconcentration than muscle tissues (e.g. Maulvault et al., 2018; Pan et al., 2018; Valdés et al., 2016; Xie and Lu, 2019). Plasma measurements, on the other hand, are not so frequent (e.g. Huerta et al., 2016; Nallani et al., 2016; Overturf et al., 2016; Santos et al., 2020), and no particular pattern could be distinguished in comparison to other tissues. Disparities in tissue bioconcentration are evident, and these results reveal how tissue selection may play a key role in standardization across studies. For 10 out of 22 neuroactive compounds, available BCFs were limited to a single value or tissue (e.g. morphine, mianserin, paroxetine, propofol, methylphenidate, bupropion, clobazam and bromazepam). Moreover, preliminary analysis of whole-individual BCF values of neuroactive pharmaceuticals appear to underestimate bioconcentration in fish, as a result of whole-body dilution (e.g. Kirla et al., 2016; Nakamura et al., 2008). Overall, likely underestimation of bioconcentration might be anticipated for most compounds based on available data, especially those of exclusive muscle and whole-body measurements, which will likely result in underrated risk characterization, as previously pointed out by Miller et al. (2018).

In the particular case of neuroactive pharmaceuticals, it seems fundamental that bioconcentration assessment includes fish brain, as the main drug target, which was only the case for 17% of BCF values. Also, the measurement of pharmaceutical concentrations in fish plasma is considered a highly valuable approach, establishing the link between internal concentrations and toxicological effects in fish, by comparing them to human therapeutic plasma concentrations through the Fish Plasma Model (Huggett et al., 2003). Yet, only 10% of BCFs have been

determined in plasma, and only a minute portion of studies have measured bioconcentration and toxic effects simultaneously, hampering the applicability of such approach.

Unbalanced BCFs data was also evident considering single species and single life-stages assessments, with 13 and 14 out of 22 compounds, respectively, and only three studies considered more than one fish species (Heynen et al., 2016; Nallani et al., 2016; Pan et al., 2018). Pan et al. (2018) determined fluoxetine BCF in brain and muscle of both *Carassius auratus* juveniles and *Danio rerio* adults, where after 144 h of exposure to 0.1 µg/L of fluoxetine, species showed BCF differences up to 34 times in brain and 11 times in muscle. Heynen et al. (2016) determined oxazepam BCF in *Perca fluviatilis* muscle and in *Pungitius pungitius* whole-body tissues, both juveniles, finding that BCF was 1.3 times lower in *P. pungitius*. Nallani et al. (2016) determined clozapine BCF for both channel fish juveniles and fathead minnow adults, for acute (7 days) and chronic (28 days) exposures, respectively. Though only a few between-species comparisons exist, differences in other experimental factors are pervasive (such as life-stage, tissue, or exposure-time), thus limiting direct comparisons. Furthermore, the only study comparing bioconcentration within different life-stages of the same species, i.e. *Danio rerio* adult and embryo (Pan et al., 2018), reported that after 72h of exposure to 0.1 µg/L of fluoxetine, embryos had a BCF of 0.22, whereas for adults, values ranged between 5.6 and 10.9, depending on the tissue considered. Organism size and metabolic rates are known to influence bioconcentration rates and are particularly determined by different species and life-stages (Arnot and Gobas, 2006).

Notably, highly variable BCF values highlight the complex nature of neuroactive pharmaceutical uptake in fish, the critical influence of environmental conditions, as well as tissues and species life-stage considered. Although scarce studies corroborate the influence of such parameters, our results evidence how sampling design influences bioconcentration of neuroactive compounds and consequently toxicity evaluation, pointing to the absolute need for higher standardization in future environmental risk assessment studies, particularly for improving accuracy and appliance in future regulatory legislation.

Bioconcentration and toxicity

Uptake and accumulation of chemical compounds in aquatic organisms is generally considered an hazard measure itself in ecotoxicity studies, independently of any existing acute or chronic effects (van der Oost et al., 2003). Notwithstanding, effects are expected after increasing internal concentrations up to a certain threshold - the internal critical concentration (Sijm and Hermens, 2005). For pharmaceuticals, and according to the read-across hypothesis

(Huggett et al., 2003; Rand-Weaver et al., 2013), biological effects can be expected if human drug targets are conserved in biota, which is frequently the case in fish, particularly *Danio rerio*, sharing more than 90% of human drug target orthologues (Gunnarsson et al., 2019). Hence, the internalized pharmaceutical concentration should dictate first a pharmacological and later a toxicological response in fish, if human therapeutic concentrations are reached (Huggett et al., 2003). Yet, despite the increasing number of studies focusing on the toxicity of pharmaceuticals in fish, few have simultaneously determined the internalized concentration of the compound, making it difficult to establish a link between bioconcentration and toxicity for many compounds (McCarty et al., 2011; Rand-Weaver et al., 2013). In this study, we show that neuroactive pharmaceuticals are not an exception, with only a very small percentage of studies (10.4 %) measuring both bioconcentration and toxic effects.

Our results show that neuroactive pharmaceutical bioconcentration was highly correlated to fish lethality, evidencing that neuroactive compounds with increasing bioconcentration are more lethal to fish (have lower LC50). For instance, the compound with lower median BCF, morphine, had a LC50 of 52,509 µg/L, whereas the two compounds with higher BCF, sertraline and mianserin, showed median LC50 of 0.31 and 6.56 µg/L, respectively. This is in agreement with the concept of lethal body burden, where for any specific chemical, when a particular internal effect concentration - the lethal body burden - is reached, the death of an organism is ascertained (Sijm and Hermens, 2005).

On the other hand, no correlations were found for all other endpoints considered, i.e. available data do not suggest that neuroactive compounds that bioconcentrate more increase the likelihood of effects on fish growth and condition, behaviour or reproduction. While at least for behavioural effects a significant correlation would be expected, considering neuroactive compounds modes of action, for reproductive, growth or condition endpoints, lack of correlation could be the result of a less straightforward link to toxicity, and thus not result in direct effects in fish (Kreke and Dietrich, 2008; Lopes et al., 2020). However, considering the fact that very few studies have confirmed pharmaceutical internal concentrations, makes the comparison less straightforward. Overall, the absence of correlations between increasing bioconcentration potential and effects likely results from insufficient and highly variable data, allied to the indirect comparison of a median BCF and minimum response concentration (MRC) values originated from different studies. Accordingly, when looking into the particular cases where both assessments were conducted, it becomes evident that, for each pharmaceutical independently, concentrations at which significant effects are observed relate to increased internal concentrations, highlighting the need for such an approach in future studies.

Specifically, in behavioural studies, 25 out of 29 reported significant effects following exposure to neuroactive pharmaceuticals, yet only 5 measured bioconcentration and behavioural changes (predatory avoidance and feeding) simultaneously (Cerveny et al., 2020; Heynen et al., 2016; Hubená et al., 2020; Xie et al., 2015; Xie and Lu, 2019). A higher number of studies on the effects to fish growth and condition were found (47 studies), although significant effects were only observed in 16 studies, and only in 3 were internalized concentrations measured (Duarte et al., 2020; Hubená et al., 2020; Pan et al., 2018). For fecundity and hatching responses, of the 24 studies that assessed the effects of exposure to neuroactive compounds, 13 reported significant effects, yet none measured internal concentrations at the time significant effects were observed.

Overall, the scarcity and heterogeneity of available data are likely behind the lack of correlation between bioconcentration potential and the toxicity of neuroactive compounds. Accordingly, when analysing the few studies where both assessments were considered, results evidenced that significant effects are linked to higher bioconcentration levels of individual neuroactive pharmaceuticals, emphasizing the benefits of such conjugated approach.

Biological effects and environmental concentrations

For 18 neuroactive pharmaceuticals, significant effects have been reported for either behaviour (predatory avoidance and feeding), growth and condition, reproduction (fecundity and hatching) or lethality (LC50). For the majority of compounds, either behavioural or growth and condition endpoints were, whenever available, the most sensitive endpoints (having lower MRC), except for metamfetamine, for which reproduction was found to be affected at lower concentration (0.597 µg/L, Liao et al. (2015)), than condition (1.1 µg/L, Hubená et al. (2020)). This is in agreement with previous studies, where behaviour has been pointed out as the most sensitive endpoint to study the effects of various contaminants in fish, including psychoactive compounds, in comparison to reproduction, development or lethality (Cunha et al., 2019; Melvin and Wilson, 2013). Still, for 4 compounds only one type of endpoint was available (paroxetine, bromazepam, clobazam and clozapine), and for 3 other compounds (diazepam, morphine and propofol) only LC50 values were available for MRC determination, due to both lack of significant responses and lack of tests. For instance, diazepam exposure did not affect the fecundity of *Pimephales promelas* adults up to 13.4 µg/L (Lorenzi et al., 2014) or hatching of *Danio rerio* embryos up to 88 µg/L (Kalichak et al., 2016), whereas no studies exist for morphine and propofol. Likewise, effects on predatory avoidance or feeding behaviours have not been studied for any of the three compounds, whereas for growth and condition, no changes

were observed in *Oreochromis niloticus* adults after 3 h of exposure to propofol (Valença-Silva et al., 2014) and no information is available for morphine and diazepam.

Considering environmental concentrations of neuroactive pharmaceuticals in the context of minimum response concentrations (MRC), the most toxic compounds in freshwater environments were antidepressants. Low MRCs in combination with rather high concentrations in surface waters resulted in 5 out of 8 antidepressants found at concentrations either above or close to fish MRC. In particular, fluoxetine was found in surface freshwaters at median concentrations 2.2 times higher than the MRC for predatory avoidance changes in fish, 0.008 µg/L (Martin et al., 2017). Critically, the highest environmental concentrations reported in these environments for antidepressants fluoxetine and citalopram largely exceeds behavioural MRC, over 800 times for citalopram (maximum reported value of 76 µg/L) and over 5000 times for fluoxetine (maximum reported value of 43 µg/L). Also, the presence of antidepressants amitriptyline, sertraline and venlafaxine and psychostimulant metamfetamine in freshwater environments is imminently critical, considering that current maximum reported concentrations are just a few decimals of µg/L from potentially affecting fish growth and condition, behaviour or reproduction. Antiepileptic carbamazepine and antipsychotic clozapine are also critical, with maximum water concentrations exceeding MRC for growth and condition (1 µg/L Qiang et al. (2016), and 30.8 µg/L Overturf et al. (2012)), by 17 and 2.5 times, respectively. On the other hand, anxiolytics bromazepam and clobazam, as well as sedative temazepam, all benzodiazepines, showed relatively similar toxicity (MRC between 6.9 and 9.1 µg/L, Cerveny et al. (2020)) and are usually found at concentrations from 360 up to thousand times lower than the MRC, thus unlikely to affect fish in freshwater systems. The only exception is oxazepam, to which MRC is slightly lower, 1.8 µg/L (Brodin et al., 2013), and environmental concentrations are relatively higher, in both freshwater and brackish/marine systems. For diazepam, MRC corresponded to fish lethality (at 12700 µg/L, Nunes et al. (2005)), but previous studies showed that it is not likely to affect fish fecundity or hatching at current environmental concentrations (Kalichak et al., 2016; Lorenzi et al., 2014).

In brackish and marine environments, antidepressants were also the most critical pharmaceutical group. Akin to freshwater environments, fluoxetine was the most toxic neuroactive pharmaceutical, where MRC for fish behaviour (0.008 µg/L) equals median environmental concentration whereas it is exceeded 11 times by the maximum reported value (0.09 µg/L). Maximum concentrations reported for antidepressants citalopram (0.093 µg/L) and sertraline (0.304 µg/L), as well as for antiepileptic carbamazepine (1.41 µg/L) and anxiolytic oxazepam (2.18 µg/L) were all above fish MRC. However, these results need to be considered with caution,

given the clear information gap on species responses to neuroactive pharmaceuticals under brackish and marine experimental conditions. Out of the 18 compounds considered, only 4 MRC values were available, and following a more conservative approach to MRC selection, the most sensitive data available were largely from freshwater studies (17 compounds). Moreover, information on occurrence of neuroactive pharmaceuticals is far less common for brackish and marine systems than for freshwater, although the increased effort towards coastal and marine systems noticed in recent years (Fonseca and Patrick, 2019; Gaw et al., 2014). For instance, the presence of four neuroactive compounds in brackish and marine systems is still unknown, namely mianserin, clobazam, clozapine and propofol, the former equally lacking occurrence data for freshwater systems.

Overall, in light of current environmental concentrations, antidepressants, antipsychotics and antiepileptics can be regarded as the most critical neuroactive pharmaceutical classes, as most compounds are currently present in aquatic systems at concentrations known to affect mostly fish behaviour or their growth and condition. This is especially critical for freshwater systems, but also for brackish and marine systems, where for instance, antidepressant sertraline and anxiolytics bromazepam and oxazepam, reach higher concentrations than in freshwater environments. With predicted increase in environmental concentrations by a growing world population and improved access to medical care (Kuster and Adler, 2014), the imminent risk is likely to occur through exposure to several more neuroactive pharmaceuticals, which are, at this point, almost reaching fish MRC for behaviour, growth and condition and reproduction.

Conclusion

Despite the increasing number of studies on the effects of neuroactive pharmaceuticals in fish over the last decades, the majority of all 451 publications focused mainly on antidepressants (largely fluoxetine), anesthetics used in aquaculture (tricaine mesylate and benzocaine) and the antiepileptic carbamazepine, accounting for more than half of all studies. Moreover, the vast majority (86%) of studies encompasses freshwater conditions, and 216 publications targeted only four freshwater species. Results also evidenced limited biological and environmental data on neuroactive pharmaceuticals in brackish and marine species and environments. Although contaminant uptake and bioconcentration are considered hazard measurements in ecotoxicology, and their estimation used for establishing thresholds in regulatory chemical and environmental legislation, it is seldomly considered in studies. It covers only 22 out of all 87 neuroactive compounds studied, being determined in less than 15% of all studies.

Overall, we found a weak link between BCF and neuroactive compound lipophilicity ($logK_{ow}$), and further emphasized the apparent misclassification of some neuroactive pharmaceuticals bioconcentration under currently established regulatory thresholds ($logK_{ow} > 3/4.5$). Still, substantial variability in bioconcentration (BCF) was evident for many compounds, associated with between-studies variability, including 67% of variation linked to abiotic parameters (temperature, exposure time, salinity and pH), and also potential inter-species, life-stages and tissues differences. The influence of sampling design is highlighted, pointing to the absolute need for higher standardization in future studies, for improved calibration and consequently environmental risk assessment.

The available data support higher lethality of neuroactive pharmaceuticals with higher bioconcentration. However, no other correlations were observed when considering effects on growth and condition, behaviour or reproduction, likely as a result of highly variable and insufficient data. The lack of studies simultaneously determining bioconcentration and toxicity effects hinders direct comparison, highlighting the importance of measuring internalized concentrations and toxicity effects simultaneously.

In the context of environmental risk posed by neuroactive pharmaceuticals, nine compounds (fluoxetine, citalopram, sertraline, amitriptyline, venlafaxine, clozapine, carbamazepine, metamfetamine and oxazepam) are either critically exceeding or imminently reaching toxic concentrations for fish in freshwater or brackish and marine environments. Allied to the expected increase of environmental concentrations it is paramount that these nine compounds are reviewed and prioritized within risk assessment studies, and included in risk management strategies and regulations, such as the watch list under the Water Framework Directive of European Commission.

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CHAPTER 3

Neuroactive pharmaceuticals in estuaries: occurrence and tissue-specific bioaccumulation in multiple fish species

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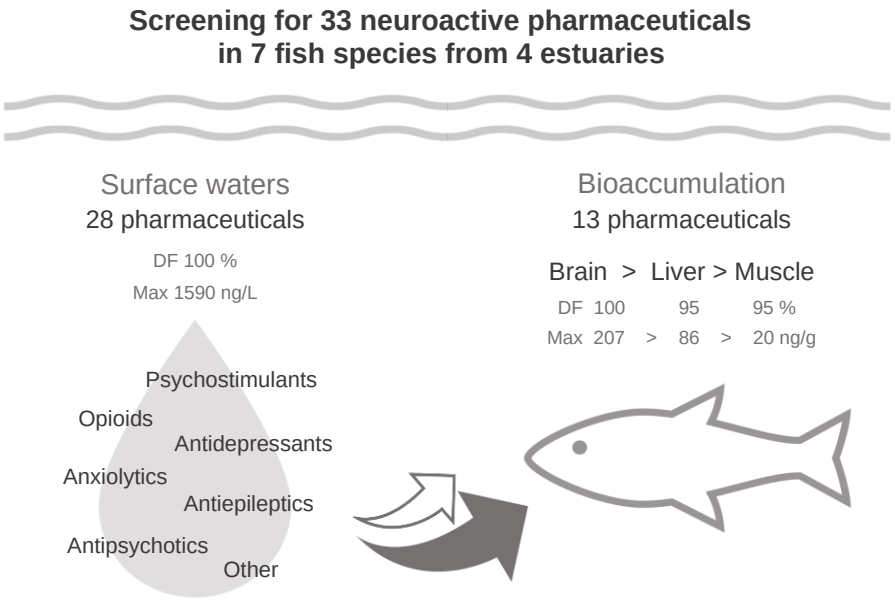

Abstract

Contamination of surface waters by pharmaceuticals is an emerging problem globally. This is because the increased access and use of pharmaceuticals by a growing world population lead to environmental contamination, threatening non-target species in their natural environment. Of particular concern are neuroactive pharmaceuticals, which are known to bioaccumulate in fish and impact a variety of individual processes such as fish reproduction or behaviour, which can have ecological impacts and compromise fish populations. In this work, we investigate the occurrence and bioaccumulation of 33 neuroactive pharmaceuticals in brain, muscle and liver tissues of multiple fish species collected in four different estuaries (Douro, Tejo, Sado and Mira). In total, 28 neuroactive pharmaceuticals were detected in water and 13 in fish tissues, with individual pharmaceuticals reaching maximum concentrations of 1590 ng/L and 207 ng/g ww, respectively. The neuroactive pharmaceuticals with the highest levels and highest frequency of detection in the water samples were psychostimulants, antidepressants, opioids and anxiolytics, whereas in fish tissues, antiepileptics, psychostimulants, anxiolytics and antidepressants showed highest concentrations. Bioaccumulation was ubiquitous, occurring in all seven estuarine and marine fish species. Notably, neuroactive compounds were detected in every water and fish brain samples, and in 95% of fish liver and muscle tissues. Despite

variations in pharmaceutical occurrence among estuaries, bioaccumulation patterns were consistent among estuarine systems, with generally higher bioaccumulation in fish brain followed by liver and muscle. Moreover, no link between bioaccumulation and compounds' lipophilicity, species habitat use patterns or trophic levels was observed. Overall, this work highlights the occurrence of a highly diverse suite of neuroactive pharmaceuticals and their pervasiveness in waters and fish from estuarine systems with contrasting hydromorphology and urban development and emphasizes the urgent need for toxicity assessment of these compounds in natural ecosystems, linked to internalized body concentration in non-target species.

Introduction

Pharmaceuticals include a complex variety of compounds with different chemical and therapeutic properties that are used to treat a myriad of health conditions and have greatly improved the global public health. However, the excretion of pharmaceuticals from the human body contributes to their release into wastewaters and subsequently into the aquatic environment, through wastewater effluents, including discharges from wastewater treatment plants (Adeleye et al., 2022; Arnold et al., 2014; Monteiro and Boxall, 2010; Wilkinson et al., 2022). Overall, the main input of pharmaceuticals to aquatic systems includes domestic discharges, yet industrial and hospital effluents as well as other input sources such as pharmaceuticals applied in livestock or aquaculture are also substantial (Arnold et al., 2014) and are occasionally associated with extremely high environmental concentrations, including in the mg/L range close to production sites (e.g. Fick et al., 2009; Larsson, 2014). Furthermore, pharmaceutical prescription has increased worldwide in recent years, and this trend is expected to continue, associated with population growth and higher demand and access (Arnold et al., 2014; Bernhardt et al., 2017). Accordingly, many pharmaceutical compounds are frequently detected in wastewaters and end up being detected in various environmental matrices, generally at low μg/L concentrations, such as surface and ground waters or sediments, depending on the pharmaceutical chemical properties and their fate in the environment (aus der Beek et al., 2016; Fatta-Kassinos et al., 2011; Wilkinson et al., 2022; Zhou et al., 2019).

The recognition that pharmaceuticals target evolutionarily conserved pathways among humans and other animals (Gunnarsson et al., 2008) and elicit effects at very low dosages, has spurred interest in the evaluation of environmental concentrations and their potential effects across fish and aquatic invertebrate species (Corcoran et al., 2010; Fabbri and Franzellitti, 2016; Mezzelani et al., 2018). Of the variety of biological effects explored, development, reproductive

and behavioural impacts, have been reported in a variety of species and in some cases depicting deleterious effects at environmentally relevant concentrations (Corcoran et al., 2010; Duarte et al., 2022; Fabbri, 2015). Moreover, the potential toxicity and pervasiveness of pharmaceutical compounds in the natural environment worldwide have led to their inclusion in European legislation as emerging contaminants of priority concern, with the potential to threaten aquatic ecosystems and human health (European Parliament and Council, 2013). In this context, selected pharmaceutical compounds, including neuroactive pharmaceuticals such as venlafaxine and its metabolite O-desmethylvenlafaxine, have been recommended for broad-scale assessment in monitoring programmes (European Commission, 2022).

Neuroactive pharmaceuticals target the central nervous system through different modes of action aiming to treat a variety of human conditions, such as depression, anxiety or epilepsy, among many others. Therefore, the continued exposure of fish to neuroactive compounds is expected to elicit responses and effects on sub-individual physiological processes, as well as higher-level individual and ecological impacts such as changes to fish behaviour, growth and reproduction that may threaten fish populations (Bertram et al., 2022; Brodin et al., 2014; Calisto and Esteves, 2009; Hamilton et al., 2016; Melvin and Wilson, 2013). In toxicity studies, molecular effects are among the most frequently assessed, followed by alterations in fish behaviour, physiology and growth, whereas studies on behavioural changes are pointed as of higher sensitivity (Duarte et al., 2022; Melvin and Wilson, 2013). However, reported effects are manifold, and their direction and intensity seem to vary within different species or life-stages (Calisto and Esteves, 2009; Cunha et al., 2019, 2017; Duarte et al., 2022; Sehonova et al., 2018), and generally lack the simultaneous link to internalized tissue concentrations (Duarte et al., 2022; Miller et al., 2018).

Pharmaceutical bioaccumulation has been shown for many therapeutic classes such as antibiotics, antidepressants, among many others, in both invertebrates and vertebrates (Mezzelani et al., 2018; Silva et al., 2015; Świacka et al., 2022). In the particular case of fishes, the bioconcentration of neuroactive pharmaceuticals has been reported for many compounds under controlled laboratory conditions (Duarte et al., 2022) but also in wastewater-impacted aquatic systems (e.g. Arnnok et al., 2017; Grabicova et al., 2017; Lahti et al., 2011). Prediction of neuroactive compounds' bioconcentration, even under controlled conditions, does not seem to be straightforward or directly correlated to the compound's physical and chemical properties, since a multitude of factors such as species, life-stages or tissues, etc, have a large impact (Duarte et al., 2022). Further environmental assessments are needed to fully understand the complexity of neuroactive pharmaceuticals accumulation in fish. In particular, the bioaccumulation patterns

in natural aquatic systems are still seldomly explored, and includes mostly freshwater environments (Gaw et al., 2014; Mezzelani et al., 2018; Świacka et al., 2022). Although less studied compared to freshwater systems, estuarine and coastal areas are highly impacted systems, and as a result of the settlement of almost half of the world's population, they are also the major recipients of urban effluents and therefore of many pharmaceutical residues (Fonseca and Reis-Santos, 2019; Martínez et al., 2007). These areas encompass a variety of habitats, supporting the life and development of numerous species, including many fish species that depend on these systems to complete their life cycle. Therefore, a few recent studies have explored pharmaceuticals bioaccumulation in coastal systems, pointing to the importance of assessing their impacts in non-target species (e.g. Ali et al., 2018; Fonseca et al., 2021), yet scarcely any have explored patterns considering a multi-species, multi-tissue and multi-system approach, towards unravelling the fate and potential risk of neuroactive pharmaceutical compounds.

Here, we present a comprehensive assessment of neuroactive pharmaceutical occurrence in surface waters and in wild biota from various estuarine systems along the North Atlantic Portuguese Coast, aiming at further understanding the fate of potentially toxic neuroactive pharmaceuticals, including their bioaccumulation in different organs (muscle, liver and brain) of several fish species, with different life-history strategies and varying habitat use patterns of estuarine systems.

Materials and Methods

Sampling areas

Four Portuguese estuaries with different morphological features and distinct levels of anthropogenic pressures, including resident population and wastewater-related parameters were sampled (Douro, Tejo, Sado and Mira) (Figure 3.1 and Table 3.1). Douro estuary, located in the north of Portugal, is surrounded by the second most populated metropolitan region in Portugal with a resident population of ca. 0,68 million inhabitants in the vicinity of the watershed. It’s an estuary with a relatively low area and volume, with a high river flow (Table 3.1). The Tejo estuary is surrounded by the most populated metropolitan area in the country (ca. 1.84 million inhabitants in the municipalities surrounding the Tejo watershed), and is the largest estuary in Portugal, characterised by large area and volume (Table 3.1). The Sado estuary, located south of Tejo, is the second largest estuary in the country, surrounded by less populated areas (ca. 0.26 million inhabitants) than Douro and Tejo, whilst the Mira estuary is the smallest

of the estuaries with a smaller area, volume and river flow, and is surrounded by a smaller resident population (ca. 0.02 million inhabitants) (Table 3.1).

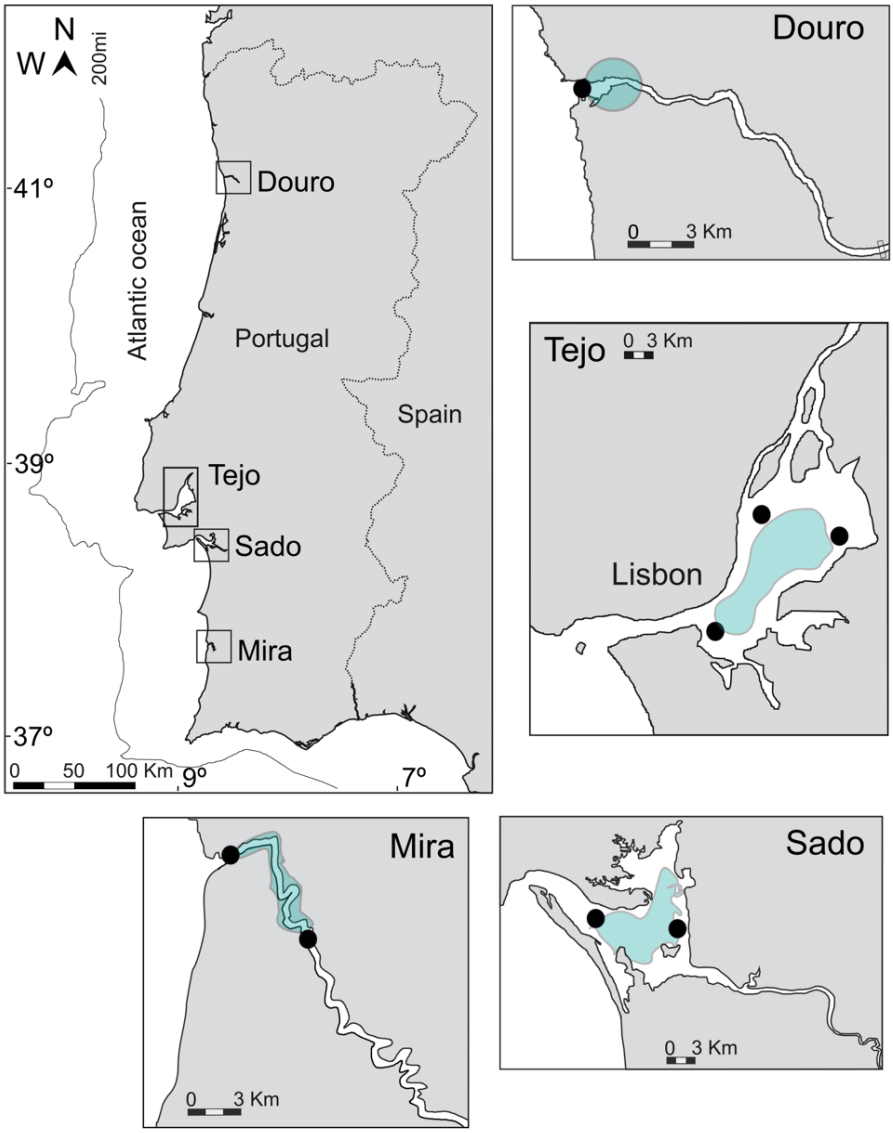

Figure 3.1. Sampled estuarine areas (Douro, Tejo, Sado and Mira estuaries), where water (black circles) and fish (coloured areas) samples were collected.

Secondary and tertiary treatments are applied to a considerable portion of all wastewater volume treated across the four estuaries (>73%, Table 3.1), with the proportion of dwellings served by wastewater drainage on average higher in Douro and Tejo estuaries (above 90%), followed by Sado (88%) and Mira (66%) estuaries.

Table 3.1. Geomorphologic and hydrologic features of all four estuarine systems (Douro, Tejo, Sado and Mira). Resident population and information concerning wastewater treatment in the surrounding municipalities are presented.

	Unit	Treatment level	Douro	Tejo	Sado	Mira
Total area [1]	km^2		10	320	180	5
River flow [1]	m^3s^{-1}		450	300	40	3
Mean depth [1]	m		4	5	6	4
Residence time [1]	days		2	25	30	15
Volume [1]	10^6 m^3		59	1900	500	27
Resident population [2]	Nº [A]		683063	1840523	257551	24717
Percentage of wastewater volume treated in each treatment level [2]	% [A]	Primary	0	27	0	0
		Secondary	50	57	29	32
		Tertiary	50	16	71	68
Percentage of dwellings served by wastewater drainage [2]	% [B]		94	96	88	66

[1] From França et al., (2009)
[2] Statistical data by geographic location (municipalities) in the vicinity of each estuary was obtained through Statistics Portugal (www.ine.pt). The values presented correspond to the sum (A) or average (B) of all municipalities' data from 2019 for each estuarine area.

Sample pre-treatment and chemical analysis

Fish and water samples were collected in the four estuaries (Figure 3.1) during the summer of 2019. Fish species were collected with beam trawls and transported on ice into the laboratory. Individual total length and weight were recorded, and portions of dorsal muscle, liver and brain were collected from a total of 55 fish from seven different species, namely estuarine resident (ER) Lusitanian toadfish *Halobatrachus didactylus*, and marine migrants (MM) and stragglers (MS) namely the European sea bass *Dicentrarchus labrax*, the Senegal sea bream *Diplodus bellottii*, the white sea bream *Diplodus sargus*, the gilthead sea bream *Sparus aurata*, the European flounder *Platichthys flesus* and the common sole *Solea solea* (Table 3.2). Tissue samples were weighted (0.10 ± 0.01 g) and stored frozen until extraction following McCallum et al. (2019). Extraction was performed twice with 1.5 mL of acetonitrile, including tissue disruption with zirconium beads for 4 minutes at 3450 oscillations per minute (Mini Beadbeater, Biospec). After centrifugation at 17500 g for 10 min at 4 ºC (Beckman Coulter Microfuge 22R Centrifuge), the supernatant was recovered and the entire process was repeated, making a final extract volume of 3 mL per sample.

Table 3.2. Summary of fish morphometric and ecological traits from all seven species collected in the four sampled estuaries (Douro, Tejo, Sado and Mira), namely number of individuals (N), mean (and standard deviation) of total length (in mm) and weight (in g). Also shown is the size at maturity (in mm), species ecological guilds (EG) based on life cycle and estuarine habitat use (ER – Estuarine Resident, MM – Marine migrant, MS – Marine straggler) as well as the trophic levels (TL).

Estuary	Species	N	Length (mm)	Weight (g)	Size at maturity (mm)	EG[a]	TL[b]
Douro	*Dicentrarchus labrax*	5	289.6 ± 15.9	256.2 ± 23.7	361 (230-460)[b]	MM	3.5
	Platichthys flesus	5	283.2 ± 10.4	277.3 ± 34.3	224 (140-300)[b]	MM	3.3
Tejo	*Dicentrarchus labrax*	7	337.6 ± 7.7	368 ± 27	361 (230-460)[b]	MM	3.5
	Halobatrachus didactylus	5	267 ± 20	371.1 ± 97.9	367 (321-438)[c]	ER	4.0
	Solea solea	6	204.8 ± 10.6	86.7 ± 16.4	303[b]	MM	3.2
Sado	*Dicentrarchus labrax*	1	360	536.8	361 (230-460)[b]	MM	3.5
	Diplodus bellottii	6	172.5 ± 9.4	95 ± 17.5	117[d]	MM	3.6
	Halobatrachus didactylus	6	202.2 ± 37.7	172.8 ± 86.4	367 (321-438)[c]	ER	4.0
	Solea solea	2	244 ± 36.8	130.5 ± 49.4	303[b]	MM	3.2
	Sparus aurata	1	272	334	365 (330-400)[b]	MM/MS	3.7
Mira	*Dicentrarchus labrax*	2	309.5 ± 23.3	324.9 ± 72.3	361 (230-460)[b]	MM	3.5
	Diplodus sargus	2	211.5 ± 4.9	185.2 ± 2.7	173[e]	MM/MS	3.4
	Halobatrachus didactylus	3	147.7 ± 33.4	64.8 ± 52.8	367 (321-438)[b]	ER	4.0
	Sparus aurata	4	227.8 ± 20	174.4 ± 52	365 (330-400)[b]	MM/MS	3.7

[a] Franco et al., (2008);
[b] Fishbase (www.fishbase.org);
[c] Pereira et al., (2011);
[d] Santos et al., (1998), average of sexes;
[e] *Erzini et al. (2001)* in Prista et al., (2003).

Twenty-five superficial (ca. 0.3m) water grab samples (1L) were collected by hand in pre-rinsed bottles, stored frozen and away from light. In the laboratory, samples were acidified with formic acid to pH 3, filtered through GF/C and 0.45 µm polyamide membranes and extracted using OASIS^{TM} HLB cartridges, followed by a washing step with 5 mL of methanol:water (10:90) and final elution with 6 mL of methanol. Both water and fish extracts were dried in a water bath under an N_2 stream at 30°C.

Before analysis, all extracts were reconstituted in 100 µL of methanol, transferred to glass autosampler vials, and centrifuged for 5 min at 4000 rpm (Mega Star 1.6R, VWR). An equal amount of deuterated internal standards was added to all samples before extraction. Screening for neuroactive pharmaceuticals included 33 compounds (Table A2.1), selected based on a combination of commercialization data (INFARMED, 2018), available compound library and previous detection in Portuguese waters (e.g. Reis-Santos et al., 2018). A total of seven therapeutic groups were considered and will be referred to as follows: PS - Psychostimulants, OP - Opioids, AD - Antidepressants, ANX - Anxiolytics, AE - Antiepileptics, AP -

Antipsychotics and O - Other (including one anticholinergic agent, one hypnotic and sedative and one anti-dementia drug). Pharmaceutical compound concentrations were calculated by comparison with seven-point standard curves (concentrations ranging between 1 and 250 ng/mL) with internal standards and native compounds (for more details see Appendix 2, Tables A2.1 and A2.2).

All samples were analysed through liquid chromatography–tandem mass spectrometry (LC–MS/MS) following Grabic et al. (2012). Target analytes were separated using Hypersil gold columns and analysed through triple-stage quadrupole mass spectrometer (TSQ Quantiva and Quantum Ultra EMR, Thermo Fisher Scientific) coupled with an Accela LC pump (Thermo Fisher Scientific), an aPAL HTC autosampler (CTC Analytics AG) and equipped with a heated-electrospray ionisation (HESI) ion source. Instrument set-up is described in detail in Appendix 2 (Tables A2.1 and A2.2). Briefly, heated electrospray in positive or negative ion mode was used for ionization and screening of targeted pharmaceuticals and internal standards. Injection of the mobile phase was performed regularly in the analytical runs to detect carry-over effects, and no contamination was observed in either instrumental or procedural blanks. Peak identification was performed with Xcalibur™ 4.3 software (Thermo Fisher Scientific), and results are presented as ng of pharmaceutical compound per L of water or per g of wet weight (ww) of fish tissue.

Data analyses

Pharmaceutical concentrations in water (ng/L) and biota tissues (ng/g ww) are presented as median, minimum and maximum values for each pharmaceutical, but also as the sum of concentrations (Σ) per therapeutic class and for the total concentration of all pharmaceuticals. A total concentration without caffeine is also given because caffeine consumption in Portugal is mostly unprescribed, and thus differs from all remaining compounds. Detection frequency (%) is presented as the percentage of samples with pharmaceuticals detected above the limit of quantification, out of all samples analysed.

Field-derived bioaccumulation factors (BAF, L/kg) were calculated as the ratio between pharmaceutical concentrations detected in fish tissues and the median concentrations detected in the corresponding estuarine waters. Pharmaceuticals' estimated log octanol-water partition coefficient values ($\log K_{ow}$) for uncharged molecules, were obtained via KOWWIN™ program by EPI Suite™ (Estimation Programs Interface). Correlations between neuroactive pharmaceuticals' lipophilicity ($\log K_{ow}$) and field bioaccumulation factors (BAF), as well as between

trophic levels and summed pharmaceutical concentrations, were tested through Spearman rank correlation (r) analysis, where a significant level of 0.05 was considered.

Principal Component Analysis (PCA) was performed in water and fish data (sums per therapeutic class, where values below quantification limits were replaced by LOQ/2, a common procedure applied in previous studies (e.g. Osorio et al., 2016), after normalization (water) and scaling (both water and fish data), to explore potential patterns in pharmaceutical occurrence and concentration related to estuaries and species. R software (R Core Team, 2019) was used to create all figures and to perform PCA and correlation analysis.

Results and Discussion

Neuroactive pharmaceuticals in surface waters

A total of 28 out of the thirty-three neuroactive pharmaceuticals analysed were detected in the 25 water samples (Table 3.3), with individual water samples showing a minimum of 10 and a maximum of 26 different compounds. Neuroactive compounds from all the seven therapeutic groups considered were detected in at least one of four estuaries: two opioids, three antiepileptics, four antipsychotics, four anxiolytics, eleven antidepressants, one psychostimulant and three other pharmaceuticals used in different therapeutic treatments (Table 3.3). A high detection frequency was observed for the majority of screened compounds, with several being detected in over 70% of water samples in all four estuaries, such as carbamazepine, haloperidol, oxazepam, venlafaxine, memantine and trihexyphenidyl, whilst a few others were present in every sample, namely caffeine, bupropion and zolpidem. These findings demonstrate the pervasiveness and diversity of neuroactive pharmaceuticals in the analysed estuarine waters, across four different estuaries with large differences in population density and footprint, and reflect the general trend found in previous studies across aquatic systems and other therapeutic groups (e.g. aus der Beek et al., 2016; Gaw et al., 2014; Mezzelani et al., 2018; Ojemaye and Petrik, 2019; Wilkinson et al., 2022).

Pharmaceutical concentrations ranged between 0.02 and 1590 ng/L for individual analytes, whereas the concentration of the pharmaceutical mixture (∑ Total) ranged between 26 and 3068 ng/L per sample, and between 5.3 and 2724 ng/L excluding caffeine, which is mostly a non-prescribed drug. The sum of pharmaceutical concentrations reached higher median values in the Mira estuary (277 ng/L), followed by the Douro (173 ng/L), Sado (150 ng/L) and Tejo (108 ng/L) estuaries, whereas the maximum concentrations were observed in Sado and Mira estuaries (above 3060 and 2870 ng/L, respectively). The range of concentrations for individual

neuroactive pharmaceuticals detected in this study is within the range (up to thousands of ng/L) of previously reported surface water concentrations in other European estuaries (e.g. Aminot et al., 2016; Fernández-Rubio et al., 2019; Zhou et al., 2019), albeit our study stands out by considering a broader suite of neuroactive compounds.

Despite differences in hydromorphology and population density in the vicinity of these estuarine systems, there were no clear contamination patterns regarding the presence of different therapeutic groups across the estuaries, with all therapeutic groups occurring in all estuaries (Table 3.3 and Figure A2.1). Conspicuously, the highest concentrations, for all therapeutic groups, were detected in the two less populated estuaries, Sado and Mira, in some cases exceeding thousands of ng/L (Table 3.3). There is an inherent variability associated with pharmaceutical occurrence in single event water grab samples and previous studies have shown daily, weekly and seasonal variations associated with pharmaceutical consumption and occurrence, highlighting the complexity of pharmaceutical presence in wastewaters and surface receiving waters (e.g. Aminot et al., 2016; Letsinger et al., 2019; Paíga et al., 2019; Pereira et al., 2016). Still, these values could be related to the higher mass loads detected in the southern regions of Portugal likely associated with an older population and increased seasonal population linked to tourism in the summer months (Pereira et al., 2016), on top of the lower percentage of dwellings served by wastewater treatment in the vicinity areas of these estuaries, down to 83 and 66% in Sado and Mira estuaries, respectively, compared to 94 and 96% in Douro and Tejo (Table 3.1). As shown in a previous study, reduced treatment is correlated to higher and more unpredictable releases of pharmaceuticals (Fork et al., 2021), and thus direct contributions of untreated wastewater cannot be fully discarded.

Table 3.3. Neuroactive pharmaceuticals in water samples. Median (Med), minimum (Min), and maximum (Max) concentration values (ng/L) of pharmaceutical analytes detected in surface water samples of Douro, Tejo, Sado and Mira estuaries. The sum of concentrations (∑) and detection frequency (DF, %) per therapeutic group and for all analytes are also shown. < LOQ indicates values below the Limit of Quantification (DF = 0).

		Douro		**Tejo**		**Sado**		**Mira**	
Therapeutic Group	**Pharmaceuticals**	Med (Min-Max) ng/L	DF (%) N=3	Med (Min-Max) ng/L	DF (%) N=9	Med (Min-Max) ng/L	DF (%) N=7	Med (Min-Max) ng/L	DF (%) N=6
Opioids	Buprenorphine	< LOQ		< LOQ		< LOQ		< LOQ	
	Codeine	0.5 (0.4 - 0.7)	100	0.3 (0.1 - 0.7)	67	0.6 (0.3 - 22)	100	0.7 (0.3 - 11)	100
	Tramadol	25 (22 - 28)	100	21 (18 - 33)	100	12 (11 - 1590)	57	17 (6.7 - 1557)	83
Σ Opioids		26 (22 - 29)	100	21 (18 - 33)	100	11 (0.5 - 1612)	100	15 (0.3 - 1568)	100
Antiepileptics	Carbamazepine	0.79 (0.77 - 0.83)	100	0.9 (0.6 - 1.5)	100	0.5 (0.1 - 52)	86	0.7 (0.1 - 61)	100
	Clonazepam	< LOQ		1.1 (0.7 - 4.9)	44	1.5	14	1.5	17
	Topiramate	< LOQ		< LOQ		< LOQ		15	17
Σ Antiepileptics		0.79 (0.77 - 0.83)	100	1.5 (0.6 - 5.9)	100	0.5 (0.1 - 52)	86	1.1 (0.1 - 76)	100
Antipsychotics	Chlorpromazine	< LOQ		1.4	11	2.6	14	2.2 (1 - 3.3)	33
	Clozapine	< LOQ		< LOQ		1.4	14	1.8	17
	Flupentixol	< LOQ		0.9	11	0.6	14	< LOQ	
	Haloperidol	0.6 (0.2 - 0.9)	100	0.2 (0.1 - 1.2)	78	0.1 (0.04 - 1.5)	71	0.2 (0.1 - 1.7)	100
	Levomepromazine	< LOQ		< LOQ		< LOQ		< LOQ	
	Risperidone	< LOQ		< LOQ		< LOQ		< LOQ	
Σ Antipsychotics		0.6 (0.2 - 0.9)	100	0.3 (0.1 - 1.5)	78	0.3 (0.04 - 5.5)	86	0.3 (0.1 - 6.8)	100
Anxiolytics	Alprazolam	1.6 (1.3 - 1.8)	100	1.3 (1.1 - 1.6)	33	2.3 (1.1 - 3.5)	29	4.7 (1.6 - 7.8)	33
	Bromazepam	< LOQ		< LOQ		< LOQ		< LOQ	
	Clobazam	< LOQ		< LOQ		< LOQ		< LOQ	

	Hydroxyzine	0.4 (0.2 - 0.6)	67	0.4 (0.1 - 2.1)	89	0.3 (0.1 - 0.9)	57	0.3 (0.2 - 0.8)	83
	Lorazepam	5.7 (5.6 - 6.3)	100	6.7 (5.6 - 7.7)	22	79	14	9.7 (5.7 - 73)	67
	Oxazepam	3.9 (3 - 5.5)	100	5 (1.4 - 12)	100	2.4 (1.1 - 171)	86	5.3 (2.8 - 190)	100
Σ Anxiolytics		11 (11 - 13)	100	7.1 (2 - 19)	100	3.1 (1.1 - 253)	86	12 (2.8 - 271)	100
Antidepressants	Amitriptyline	0.7 (0.7 - 0.8)	67	0.7 (0.5 - 1.6)	56	1.3 (0.6 - 65)	43	2 (1.2 - 44)	50
	Bupropion	0.7 (0.7 - 0.9)	100	0.7 (0.5 - 1.4)	100	0.4 (0.1 - 59)	100	0.6 (0.3 - 48)	100
	Citalopram	0.9 (0.7 - 2)	100	1.3 (0.8 - 4.6)	89	0.9 (0.7 - 54)	43	1.3 (1.1 - 77)	83
	Duloxetine	0.2 (0.2 - 0.3)	100	0.4 (0.1 - 1.1)	67	0.3 (0.1 - 8.3)	86	1.5 (0.3 - 7.1)	83
	Fluoxetine	< LOQ		< LOQ		14 (3.5 - 24)	29	16 (8 - 133)	50
	Maprotiline	0.5	33	0.7 (0.5 - 1)	33	0.9 (0.5 - 1.3)	29	1.5 (0.8 - 2.9)	67
	Mianserin	0.5 (0.4 - 0.7)	100	0.6 (0.2 - 2.9)	89	0.4 (0.2 - 3.2)	86	0.6 (0.3 - 3.5)	67
	Mirtazapine	< LOQ		< LOQ		31	14	61	17
	Paroxetine	< LOQ		< LOQ		9.3	14	1.7 (1.1 - 6.2)	50
	Sertraline	1.3 (1.1 - 1.5)	67	1.3 (1.1 - 1.4)	22	113	14	39 (2.4 - 76)	33
	Venlafaxine	21 (9.4 - 22)	100	8.5 (1.5 - 15)	89	6 (0.5 - 336)	100	12 (0.7 - 383)	83
Σ Antidepressants		24 (15 - 26)	100	14 (3.4 - 17)	100	10 (1.6 - 705)	100	29 (2 - 715)	100
Psychostimulants	Caffeine	113 (109 - 116)	100	62 (28 - 165)	100	123 (17 - 344)	100	157 (24 - 1003)	100
Σ Psychostimulants		113 (109 - 116)	100	62 (28 - 165)	100	123 (17 - 344)	100	157 (24 - 1003)	100
Anti-dementia drugs	Memantine	0.4 (0.3 - 0.4)	100	0.8 (0.6 - 1.6)	100	0.3 (0.1 - 93)	86	0.8 (0.3 - 62)	83
Anticholinergic agents	Trihexyphenidyl	0.2 (0.1 - 0.2)	100	0.1 (0.02 - 0.3)	78	0.2 (0.1 - 0.3)	71	0.1 (0.07 - 0.3)	83
Hypnotics and sedatives	Zolpidem	0.36 (0.36 - 0.41)	100	0.4 (0.4 - 0.6)	100	0.5 (0.4 - 2.8)	100	0.4 (0.3 - 4.2)	100
Σ Other		0.9 (0.8 - 0.9)	100	1.3 (1 - 2.3)	100	0.9 (0.6 - 96)	100	1.3 (0.5 - 66)	100

Σ Total without caffeine	63 (58 - 64)	100	42 (28 - 68)	100	27 (5.3 - 2724)	100	76 (6.2 - 2703)	100
Σ Total	173 (170 - 179)	100	108 (61 - 205)	100	150 (26 - 3068)	100	277 (30 - 2876)	100
Number of pharmaceuticals	18 (18 - 19)	100	16 (13 - 18)	100	14 (11 - 24)	100	19 (10 - 26)	100

Overall, the occurrence of pharmaceutical compounds differed among and within therapeutic groups, with psychostimulants reaching higher median concentrations, followed by antidepressants, opioids and anxiolytics (Table 3.3). The psychostimulant caffeine was found in every water sample analysed, and it was the compound with higher median concentrations in all estuaries (between 62 up to 157 ng/L), being higher in the Mira and Sado estuaries, followed by Douro and Tejo, with concentrations ranging between 16 up to 1003 ng/L (Table 3.3). Caffeine is highly consumed in Portugal as generally worldwide (e.g. Quadra et al., 2020), and although secondary wastewater treatment has been shown to reach exceptional removal efficiencies in some cases (Adeleye et al., 2022), high consumption and permanent release of caffeine seem to be contributing to its ubiquity and high concentrations in aquatic systems, reaching thousands of ng/L in marine and estuarine waters (Vieira et al., 2022), in line with those found in this study.

Within opioids, buprenorphine was not detected in any water sample, whereas codeine was detected in all water samples from Douro, Sado and Mira estuaries, at concentrations ranging from 0.1 up to 22 ng/L. Tramadol was also ubiquitous in the Douro and Tejo water samples, but not in Sado (57%) or Mira (83%) yet reaching much higher concentrations up to 1590 ng/L, in the latter. Although opioid consumption in Portugal is relatively low in comparison with other European countries (OECD, 2019), it has been increasing in recent years, with tramadol, codeine and buprenorphine being among the most prescribed opioids, for instance, in the Lisbon metropolitan area (Caldeira et al., 2021). Moreover, recent studies have shown that many opioids are not efficiently removed by wastewater treatment, some even following tertiary treatment (Asimakopoulos and Kannan, 2016; Campos-Mañas et al., 2018), resulting in its frequent detection in environmental water samples, as in our study, in particular tramadol and codeine, two of the most frequently detected opioids (Campos-Mañas et al., 2018). Likewise, buprenorphine, which was not detected in our water samples, is reported as less frequently detected in both wastewater influents and effluents, and occurring at much lower concentrations compared to codeine or tramadol (Asimakopoulos and Kannan, 2016; Campos-Mañas et al., 2018). Tramadol reached by far the highest concentrations of all three opioids considered, yet similar concentrations (reaching thousands of ng/L) have been previously reported for coastal waters (e.g. Sousa et al., 2020).

The eleven antidepressants screened for in this study were all detected in the estuarine waters. Eight of the antidepressants were found in all four estuaries, whereas the other three, namely fluoxetine, mirtazapine and paroxetine, were found exclusively in Sado and Mira waters (Table

3.3). Bupropion, venlafaxine, duloxetine and mianserin were frequently detected in all four estuaries (DF > 67%), yet reaching different maximum concentrations (Table 3.3). Higher median and maximum concentrations were observed for venlafaxine (maximum surpassing 300 ng/L in Sado and Mira), whereas sertraline and fluoxetine also reached more than 100 ng/L, despite being less frequently detected. Antidepressants are of the most widely screened and detected pharmaceutical classes, being found worldwide in a vast range of concentrations and in different environmental matrices (aus der Beek et al., 2016; Calisto and Esteves, 2009; Sehonova et al., 2018; Wilkinson et al., 2022). The concentrations found in our samples are in agreement (ca. from below tens up to hundreds of ng/L) with those previously detected in estuarine (e.g. Fernández-Rubio et al., 2019; Reis-Santos et al., 2018) and marine waters (e.g. Björlenius et al., 2018; Nödler et al., 2014; Togola and Budzinski, 2008).

Four out of six anxiolytic pharmaceuticals were detected in estuarine waters. Oxazepam was frequently found in all four estuaries (DF > 86%) at concentrations ranging from 1.1 up to 190 ng/L. This is in agreement with the concentrations reported in previous studies (ca. tens of ng/L) in riverine (e.g. Aminot et al., 2015; Fick et al., 2017; Wang et al., 2017), estuarine and sea waters (Björlenius et al., 2018; Fernández-Rubio et al., 2019), as well as being the most prevalent benzodiazepine in wastewaters (Asimakopoulos and Kannan, 2016) due to generally low removal percentage following wastewater treatment (e.g. de Boer et al., 2022; de Jesus Gaffney et al., 2017; Kosjek et al., 2012). Alprazolam, hydroxyzine and lorazepam were also present in all four estuaries, yet detection frequencies varied from 14 up to 100%, with concentrations up to 7.8, 2.1 and 79 ng/L, respectively, which have also been found in previous studies in surface waters from the Atlantic coast and other locations worldwide (aus der Beek et al., 2016; Fernández-Rubio et al., 2019; Fick et al., 2011).

Carbamazepine was the most frequently detected antiepileptic pharmaceutical in estuarine water. It was found in every sample from the Douro, Tejo and Mira estuaries, and 86% of samples in the Sado estuary, at concentrations ranging from 0.1 to 61 ng/L. Carbamazepine is a commonly prescribed antiepileptic worldwide, known to be able to resist wastewater treatment at low concentrations and is the most frequently detected antiepileptic in wastewaters and in the environment worldwide (Adeleye et al., 2022; aus der Beek et al., 2016; Cardoso-Vera et al., 2021; Zhang et al., 2008). Hence, in estuarine and coastal waters carbamazepine has been found to reach maximum concentrations of thousands of ng/L (e.g. McEneff et al., 2014), and many studies frequently report

100% detection in surface waters (Cardoso-Vera et al., 2021). Other antiepileptics analysed included clonazepam and topiramate, which were less frequently detected (up to 44 and 17%, respectively) and at concentrations up to 4.9 and 15 ng/L, respectively. Very few studies have screened for these pharmaceuticals in surface waters, so there is still limited information, though there are reports of no detection or detection at the same range of concentrations (below 20 ng/L) as found here (e.g. Pivetta et al., 2020; Renganathan et al., 2021).

Of all six antipsychotic pharmaceuticals analysed in the water, haloperidol was the most common, being present in all samples from Douro and Mira estuaries and on more than 70% of samples from Tejo and Sado systems, at concentrations ranging from 0.04 up to 1.7 ng/L. On the other hand, chlorpromazine, clozapine and flupentixol were seldom detected in the water, with frequencies between 11 and 33% in Tejo, Sado and Mira estuaries, whereas levomepromazine and risperidone were not detected (Table 3.3). Despite the presence of some of these antipsychotics in wastewater effluents (e.g. Loos et al., 2013), few studies have assessed their occurrence in surface waters, yet they are generally not detected or detected at low ng/L concentrations (e.g. aus der Beek et al., 2016; Dehm et al., 2021; Escudero et al., 2021; Kondor et al., 2020), although some exceptionally high concentrations of clozapine (up to 78 μg/L) have been observed in South Africa's Umgeni and Msunduzi rivers (Matongo et al., 2015a, 2015b). To the best of our knowledge, we present the first record of clozapine in estuarine waters.

Within the Other pharmaceutical compounds group, which includes anti-dementia drug memantine, anticholinergic agent trihexyphenidyl and hypnotic sedative zolpidem, high detection frequencies were observed, >70% for all compounds, with concentrations ranging from 0.02 up to 93 ng/L. Detected concentrations of zolpidem are in the same range of concentrations previously found in surface waters, whereas memantine reached higher concentrations than in previous studies, and trihexyphenidyl concentrations were lower than previously reported (e.g. aus der Beek et al., 2016; Brieudes et al., 2017; Dehm et al., 2021). Notwithstanding, for all three compounds, the concentrations found in this study are lower than the maximum reported in wastewaters (Fick et al., 2011; Loos et al., 2013).

Overall, a highly diverse suite of neuroactive compounds was detected in surface waters from the four estuaries. Almost half (15) of the neuroactive compounds screened were found at concentrations above the threshold defined for studies on environmental fate and effects (10 ng/L) according to the European Medicines Agency (EMA) and reaching maximum concentrations over

150 times higher. Moreover, the ubiquity and diversity of these compounds are outstanding, with more than 10 and up to 26 compounds being detected in every sample collected in distinct estuaries.

Neuroactive pharmaceuticals in fish

Thirteen out of the 33 neuroactive pharmaceuticals screened were detected in at least one of the fish tissues (i.e., brain, liver and muscle tissues), and included one opioid, two antiepileptics, two antipsychotics, two anxiolytics, four antidepressants, one psychostimulant and one compound from the Other pharmaceutical compounds group (Table 3.4). Notably, all brain samples (50) and 95% of liver and muscle samples (55 each) contained at least one neuroactive pharmaceutical. Still, in fish brain and liver tissues, a median of 2 neuroactive pharmaceuticals were detected, with individual samples showing up to 6 different compounds, whereas in the muscle samples a maximum of 3 different compounds per sample were detected (Table 3.4). Pharmaceutical concentrations ranged between 0.1 and 207 ng/g for individual analytes, with antiepileptic topiramate, antidepressant venlafaxine and the psychostimulant caffeine exhibiting the highest concentrations (Table 3.4). The sum of all neuroactive pharmaceutical concentrations ($\sum$ Total) per sample reached higher median concentrations in the brain (9 ng/g), followed by the liver (5.8 ng/g) and muscle (1.5 ng/g) tissues (Table 3.4 and Figure 3.2). The same pattern was also observed for the maximum tissue concentrations, with brain reaching 207 ng/g followed by liver 86 ng/g and muscle 21 ng/g (Table 4). Laboratory (e.g. Huerta et al., 2016; McCallum et al., 2017; Valdés et al., 2016) and field studies (e.g. Brooks et al., 2005; Liu et al., 2018) have shown similar accumulation patterns among tissues, with brain and liver tissues showing higher concentrations than muscles. These patterns and the presence of different pharmaceuticals among tissues have implications towards the choice of tissues for pharmaceutical quantification and environmental risk assessment (e.g. Duarte et al., 2022; Miller et al., 2018).

Overall, the bioaccumulation of neuroactive pharmaceuticals in fish species differed among and within therapeutic groups, with antiepileptics reaching higher summed concentrations, followed by psychostimulants, anxiolytics and antidepressants (Table 3.4 and Figure 3.2). Antiepileptics and antipsychotics were among the most frequently detected therapeutic groups, with frequencies of detection higher than 47% in all three tissues.

Table 3.4. Neuroactive pharmaceuticals in fish samples. Median (Med), Minimum (Min), and Maximum (Max) concentration values (ng/g ww) of pharmaceutical analytes detected in different fish tissues (brain, liver and muscle) collected from Douro, Tejo, Sado and Mira estuaries. The sum of concentrations (∑) and detection frequency (DF, %) per therapeutic group and for all analytes (∑ Total) are also shown. < LOQ indicates values below the Limit of Quantification (DF = 0).

		Brain		Liver		Muscle	
Therapeutic Group	**Analyte**	Med (Min-Max) ng/g	DF (%) N=50	Med (Min-Max) ng/g	DF (%) N=55[a]	Med (Min-Max) ng/g	DF (%) N=55
Opioids	Buprenorphine	< LOQ		< LOQ		< LOQ	
	Codeine	0.8 (0.5 - 1.7)	28	0.9 (0.6 - 1.6)	21	0.7 (0.7 - 1.1)	13
	Tramadol	< LOQ		< LOQ		< LOQ	
Σ Opioids		0.8 (0.5 - 1.7)	28	0.9 (0.6 - 1.6)	21	0.7 (0.7 - 1.1)	13
Antiepileptics	Carbamazepine	1.4	2	< LOQ		< LOQ	
	Clonazepam	< LOQ		< LOQ		< LOQ	
	Topiramate	12 (1.3 - 207)	72	8 (1.1 - 86)	62	2.3 (1.2 - 20)	47
Σ Antiepileptics		12 (1.3 - 207)	72	8 (1.1 - 86)	62	2.3 (1.2 - 20)	47
Antipsychotics	Chlorpromazine	< LOQ		< LOQ		< LOQ	
	Clozapine	< LOQ		< LOQ		< LOQ	
	Flupentixol	< LOQ		< LOQ		< LOQ	
	Haloperidol	< LOQ		0.2 (0.1 - 0.5)	23	< LOQ	
	Levomepromazine	< LOQ		< LOQ		< LOQ	
	Risperidone	0.2 (0.1 - 0.3)	72	0.2 (0.1 - 0.6)	69	0.2 (0.1 - 0.4)	65
Σ Antipsychotics		0.2 (0.1 - 0.3)	72	0.2 (0.1 - 0.6)	73	0.2 (0.1 - 0.4)	65
Anxiolytics	Alprazolam	< LOQ		< LOQ		< LOQ	
	Bromazepam	< LOQ		< LOQ		< LOQ	
	Clobazam	< LOQ		< LOQ		< LOQ	
	Hydroxyzine	< LOQ		1.4	2	< LOQ	

	Lorazepam	< LOQ		5.3	2	< LOQ	
	Oxazepam	< LOQ		< LOQ		< LOQ	
Σ Anxiolytics		< LOQ		6.7	2	< LOQ	
Antidepressants	Amitriptyline	< LOQ		< LOQ		< LOQ	
	Bupropion	< LOQ		0.2 (0.1 - 0.4)	10	0.1	2
	Citalopram	< LOQ		< LOQ		< LOQ	
	Duloxetine	1.6	2	1.7 (1.7 - 3)	6	< LOQ	
	Fluoxetine	< LOQ		< LOQ		< LOQ	
	Maprotiline	< LOQ		< LOQ		< LOQ	
	Mianserin	< LOQ		2	2	1.1	2
	Mirtazapine	< LOQ		< LOQ		< LOQ	
	Paroxetine	< LOQ		< LOQ		< LOQ	
	Sertraline	< LOQ		< LOQ		< LOQ	
	Venlafaxine	1.1 (0.5 - 13)	30	1.8 (0.6 - 4.3)	19	0.9 (0.5 - 2.5)	22
Σ Antidepressants		1.4 (0.5 - 13)	30	1.7 (0.1 - 6.1)	27	0.8 (0.1 - 2.5)	24
Psychostimulants	Caffeine	7.5 (5.5 - 9.7)	6	12	2	5.3	2
Σ Psychostimulants		7.5 (5.5 - 9.7)	6	12	2	5.3	2
Anti-dementia drugs	Memantine	< LOQ		< LOQ		< LOQ	
Anticholinergic agents	Trihexyphenidyl	0.13 (0.11 - 0.14)	4	0.12 (0.11 - 0.12)	4	0.2	2
Hypnotics and sedatives	Zolpidem	< LOQ		< LOQ		< LOQ	
Σ Other		0.13 (0.11 - 0.14)	4	0.12 (0.11 - 0.12)	4	0.2	2
Σ Total		9 (0.1 - 207)	100	5.8 (0.1 - 86)	95	1.5 (0.1 - 21)	95
Number of pharmaceuticals		2 (1 - 5)		2 (0 - 6)		1 (0 - 3)	

[a] The number of samples (N) varies for some of the analytes screened. For more details see Appendix 2, Table A2.3.

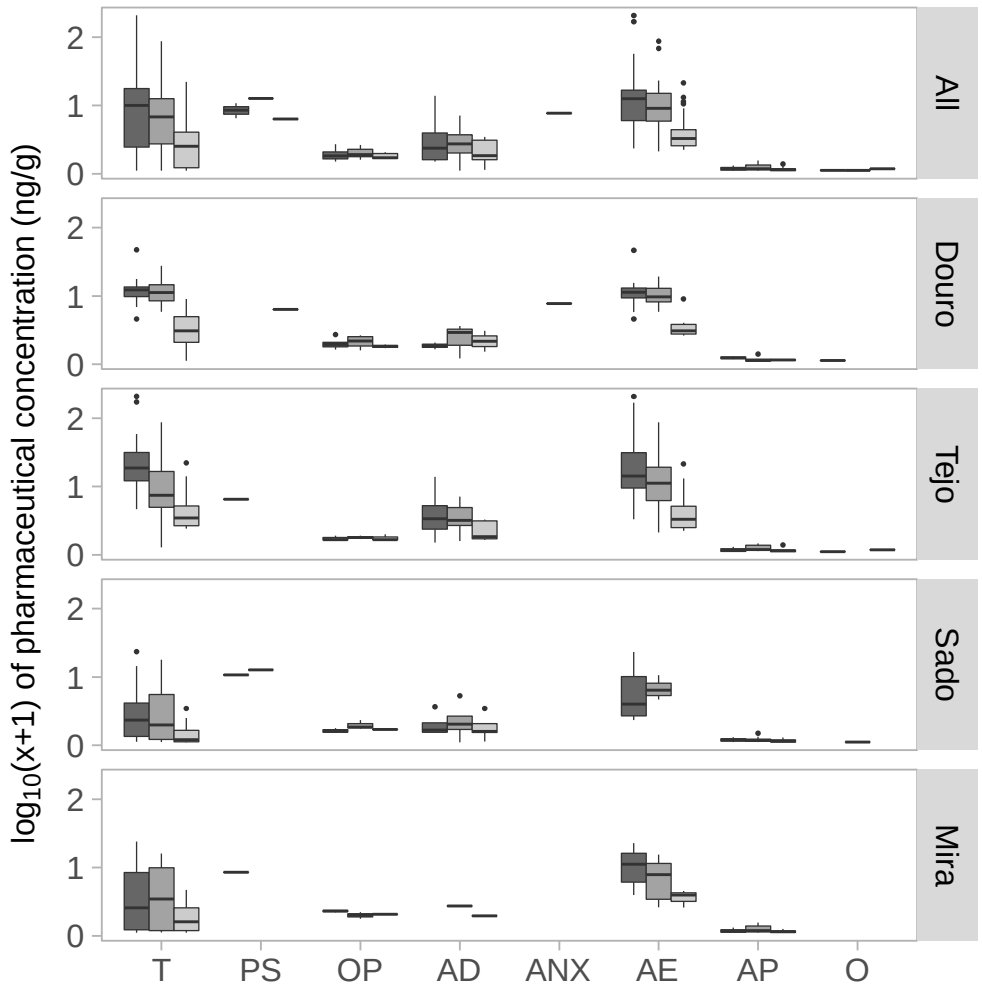

Figure 3.2. Sums (∑) of pharmaceutical concentrations per therapeutic group, in different fish tissues (brain, liver and muscle), in all and each estuary (Douro, Tejo, Sado and Mira). Values are presented as $\log_{10}(x+1)$ of pharmaceutical concentrations (ng/g ww). Left (dark grey), centre (grey) and right (light grey) boxplots correspond to brain (N=50), liver (N=55) and muscle (N=55), respectively. Boxplots show median, 25th and 75th percentiles, upper and lower whiskers extending at most 1.5 times the interquartile range (IQR) to maximum and minimum values, respectively. Therapeutic groups are the following: T - Total, PS - Psychostimulants, OP - Opioids, AD - Antidepressants, ANX - Anxiolytics, AE - Antiepileptics, AP - Antipsychotics and O - Other.

Although frequently detected in water, the antiepileptic carbamazepine was only detected in one brain sample of *P. flesus* from the Douro estuary, at 1.4 ng/g, a slightly higher value than those reported by Liu et al., (2015) and Tanoue et al., (2015) (up to 1 ng/g) in the brains of freshwater species collected in riverine systems in China and Japan, respectively. Moreover, no carbamazepine residues were previously detected in liver and muscle tissues from wild fish collected in the Tejo estuary (Fonseca et al., 2021) but have been found in wild fish species from other locations worldwide (Świacka et al., 2022). Topiramate was frequently detected in all tissues (DF > 47; brain > liver > muscle) from 5 out of 7 species, with higher median and maximum concentrations in the brain (12 and 207 ng/g, respectively), followed by liver and muscle samples (Table 3.4). In line with our results, a similar range of concentrations

has been reported in the liver of wild *D. labrax* juveniles and one adult collected in the Tejo estuary, up to 244.4 ng/g (Fonseca et al., 2021), yet whilst our results show its repeated occurrence at similar elevated levels, there is a general lack of field studies targeting this pharmaceutical in fish, and this should be prioritised, considering the high concentrations observed. Also, no bioaccumulation of the antiepileptic clonazepam was observed, and this is, to our knowledge, the first study to target this compound in wild fish, whilst studies concerning clonazepam occurrence in aqueous matrices and exposure effects are still scarce, as mentioned in recent review studies (Cunha et al., 2019, 2017).

Contrary to the high occurrence in water samples (and reaching up to 1003 ng/L), the psychostimulant caffeine was present in only 6% of fish brain samples, and 2% of both liver and muscle tissues, with concentrations between 5.3 and 12 ng/g. Other field studies also reported caffeine bioaccumulation in muscle, liver and gills of different fish species at the same magnitude (up to 74 ng/g), in wet and dry weights (Li et al., 2020; Ondarza et al., 2019; Vieira et al., 2022), yet no behavioural effects were observed at higher internal concentrations (from 29 up to 68 ng/g) in *Perca fluviatilis* juveniles (Cerveny et al., 2022) whereas changes in biochemical and behavioural endpoints were reported only at substantially higher external concentrations, above several thousands of ng/L and up to mg/L range (e.g. Ladu et al., 2015; Li et al., 2012; Santos-Silva et al., 2018).

Of the six anxiolytics analysed, only hydroxyzine and lorazepam were found, and in one liver sample of *P. flesus* from the Douro estuary, at 1.4 and 5.3 ng/g, respectively. Fonseca et al., (2021) and Huerta et al., (2018) screened for anxiolytic lorazepam in wild fish, with no detection in liver or muscle tissues in different estuarine and freshwater fish species, while Rojo et al., (2019) detected a maximum of 0.23 ng/g in the muscle of 1 out of 3 freshwater fish species. A previous study also documented low hydroxyzine uptake in liver of fish caged in a wastewater-influenced stream (0.3 up to 1.2 ng/g) and also no detection in brain and muscle tissues (Grabicova et al., 2017). Though not detected, this is, to the best of our knowledge, the first screening for clobazam in water and fish in estuarine areas. Whilst anxiolytics such as alprazolam, bromazepam were also not detected in wild fish (e.g. Fonseca et al., 2021; Martínez-Morcillo et al., 2020; Peña-Herrera et al., 2020), oxazepam bioaccumulation was reported in the plasma of wild riverine species *Squalius cephalus* at 25 ng/ml (Cerveny et al., 2021) as well as in *Perca fluviatilis'* bile below 3 ng/g (UNESCO and HELCOM, 2017).

Four out of eleven antidepressants screened were detected in fish tissues: bupropion, duloxetine, mianserin and venlafaxine. Of all four, venlafaxine was the most pervasive, and detected in 5 out of the 7 species, and in 30, 19 and 22% of brain, liver and muscle tissues

(concentrations ranging from 0.5 up to 13 ng/g). Bupropion, duloxetine and mianserin were less frequently detected, at maximum concentrations of 0.4, 3 and 2 ng/g. Antidepressants amitriptyline, citalopram, fluoxetine, maprotiline, mirtazapine, paroxetine and sertraline were not found in any fish tissues. Multiple studies have reported antidepressants' bioaccumulation in fish collected in the wild, including those screened in this study, yet with considerable variability (Miller et al., 2018; Silva et al., 2015; Świacka et al., 2022). Venlafaxine is commonly detected in wild fish, found in various tissues including brain, liver and muscles, and within the low ng/g (ww and dw) range (e.g. Arnnok et al., 2017; Huerta et al., 2018; Schultz et al., 2010). Bioaccumulation of bupropion, mianserin and duloxetine in wild fish has been previously assessed (Arnnok et al., 2017; Cerveny et al., 2021; Grabicova et al., 2017; Schultz et al., 2010), despite being seldom considered compared to other antidepressants (Silva et al., 2015; Świacka et al., 2022). On the other hand, sertraline and fluoxetine are frequently detected in wild fish tissues at concentrations below or close to our LOQ of 10 and 5 ng/g, respectively (Brooks et al., 2005; Meador et al., 2016; Schultz et al., 2010), though there are studies showing bioaccumulation up to hundreds of ng/g (e.g. Du et al., 2014; Fonseca et al., 2021; Ramirez et al., 2009). Its fast metabolism evidenced by higher concentrations of metabolites compared to the parent compounds (Arnnok et al., 2017; Miller et al., 2018; Schultz et al., 2010), in combination with the lower occurrence in our water samples might justify its absence in our fish samples, although metabolites were not screened to confirm this hypothesis. While the remaining antidepressants are comparatively less studied in wild fish, their monitoring should not be disregarded as they are frequently detected in surface waters and in wild biota (Calisto and Esteves, 2009; Silva et al., 2015; Świacka et al., 2022).

While opioids buprenorphine and tramadol were not detected in fish, codeine concentrations were generally the same across tissues, though more frequently found in fish brain followed by liver and muscle, reaching maximum concentrations of 1.7 ng/g in the brain (Table 3.4). Codeine bioaccumulation in fish has been described in various freshwater fish species collected in the field (Rojo et al., 2019; Valdés et al., 2016) and at the same range of concentrations as in this study (up to 1.1 ng/g in Rojo et al., (2019)). Tramadol has also been reported to accumulate in different fish tissues (Grabicova et al., 2017; Hubená et al., 2020; Tanoue et al., 2017), yet despite the high concentrations found in our water samples (up to 1590 ng/L), it was not detected in fish, which might be associated with the relatively high LOQ in our samples (50 ng/g) and with the different exposure conditions from our wild samples, when compared to a likely continuous exposure concentration in these studies from the experimental design in the laboratory and in caged in the field trial. In fact, Tanoue et al., (2017) and Hubená et al., (2020)

reported mean brain concentrations of 4.6 ng/g in *Pimephales promelas* adults and 1.8 ng/g in *Squalius cephalus* after long-term (23 and 42 days, respectively) exposures to 1 μg/L (the same range as some of the highest concentrations found in our water samples), whereas Grabicova et al., (2017) detected tramadol in liver and kidney of *Salmo trutta* caged for 3 months in a stream influenced by wastewater effluents, on average from 1.7 up to 6 ng/g, with all these studies pertaining to continuous exposure conditions yet all reporting concentrations below our quantification limit.

Only two antipsychotics were detected in fish: risperidone was found in all three tissues, although more frequently detected in brain (72%), followed by the liver (69%) and muscle (65%) of all species sampled, with concentrations ranging from 0.1 up to 0.6 ng/g; whereas haloperidol was found only in the liver (23%) of 5 species, at concentrations up to 0.5 ng/g. Risperidone has been frequently found in the tissues of wild fish (Cerveny et al., 2021; Grabicova et al., 2017) and fish exposed to treated effluents (e.g. Fick et al., 2010), even when, as in our study, it is not detected in the medium (Fick et al., 2010; Grabicova et al., 2017). Likewise, several studies reported haloperidol concentrations in wild fish at very low ng/g (usually below 1 ng/g or 1.2 ng/mL in plasma), such as in brain (Tanoue et al., 2015), blood plasma (Cerveny et al., 2021; Fick et al., 2010), liver or muscle (Tanoue et al., 2015). Accordingly, Tanoue et al., (2015) have shown a higher partition of haloperidol in the liver than any other fish tissue analysed, including brain and muscle, which is in line with the detection of this pharmaceutical only in the liver. Both Cerveny et al., (2021) and Fick et al., (2010) studies pointed risperidone and haloperidol as of high risk for fish, as concentrations in fish plasma were either above or close to human therapeutic plasma concentrations, implying potential exposure effects. Our results corroborate these studies, as these were the only two out of six antipsychotics to bioaccumulate in fish, even when it was not detected in the medium as observed for risperidone.

Bioaccumulation in fish of the pharmaceutical compounds considered within the Other compounds group was only observed for trihexyphenidyl (4% of samples), with concentrations ranging from 0.11 to 0.2 ng/g, lower than those previously reported in *P. flesus* from the baltic sea (UNESCO and HELCOM, 2017).

Overall, bioaccumulation patterns of the different therapeutic groups in fish were consistent among all four estuaries (Figure 3.2). Generally larger contributions for summed concentrations were from antiepileptics, psychostimulants, anxiolytics and antidepressants groups (Table 3.4 and Figure 3.2), which follows patterns in previous studies. For example, Muir et al., (2017) screened for 127 pharmaceuticals and personal care products in the plasma of both

caged *Carassius auratus* and wild *Cyprinus carpio*, with more than half of the compounds detected in fish tissues being antidepressants and their metabolites. Following the screening of 20 pharmaceuticals in 8 different fish species, Huerta et al., (2018) also found antiepileptics and antidepressants to be the most prevalent therapeutic groups among the seven groups considered, including for example β-blockers or anti-inflammatory drugs. In their work, Arnnok et al., (2017) screened for 24 pharmaceutical compounds in 10 different fish species and also found higher concentrations of antidepressants compared to other classes of pharmaceuticals such as antibiotics or anti-inflammatory drugs.

Although variations in occurrence and concentrations were observed in the waters from the four estuaries, bioaccumulation patterns were similar in fish from all the systems (Figure 3.2). This has also been observed in other field studies where sampling occurred in various locations, suggesting that bioaccumulation patterns are mostly determined by the chemical properties of pharmaceuticals rather than the range of concentrations found in the medium (e.g. Muir et al., 2017; Yang et al., 2020). Despite maximum water concentrations found in Sado and Mira estuaries (the two less populated estuaries, despite seasonal variability), fish accumulated higher levels of pharmaceuticals in Douro and Tejo estuaries, evidencing the impacts of the constant pharmaceutical inputs from highly populated areas. Usually, bioconcentration of lipophilic compounds is estimated through the octanol/water partition coefficient (K_{ow}) and has been shown to increase with increasing lipophilicity for different chemicals (Arnot and Gobas, 2006; Bintein et al., 1993; Mackay, 1982). Accordingly, lipophilicity thresholds have been set to estimate chemical bioaccumulation and to determine the need for environmental risk assessment of chemical substances in European guidelines. However, in the particular case of neuroactive pharmaceuticals, it seems that this factor alone may not be the best predictor for bioaccumulation in fish tissues, as their uptake and bioconcentration is influenced by parameters such as salinity, pH or exposure time, but also by species-specific traits, life-stage or tissues (Duarte et al., 2022). Accordingly, we tested if a correlation between field-derived bioaccumulation factors (BAF) and compounds' lipophilicity existed, and no significant correlation was found when considering all BAF values ($r = 0.2$, p-value = 0.53, Figure 3.3), nor when considering each tissue independently ($r > 0.2$, p-value > 0.3), confirming that the prediction of bioaccumulation of neuroactive compounds through compounds' lipophilicity may not be straightforward.

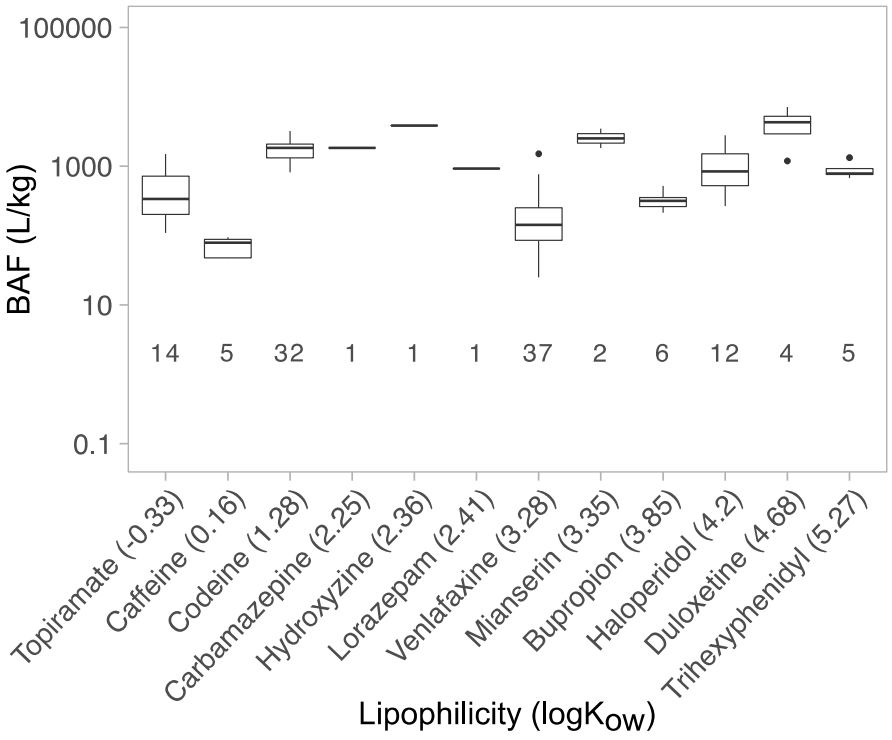

Figure 3.3. Field-derived bioaccumulation factors (BAF, L/kg) of neuroactive pharmaceuticals with increasing lipophilicity ($logK_{ow}$). BAF values (N shown under each boxplot) were calculated as the ratio between pharmaceutical concentrations detected in fish tissues and the median concentrations detected in the corresponding estuarine waters. Boxplots show median, 25th and 75th percentiles, upper and lower whiskers extending at most 1.5 times the interquartile range (IQR) to maximum and minimum values, respectively.

Neuroactive pharmaceutical bioaccumulation showed a prevalent pattern among species, with higher summed concentrations in the brain followed by the liver and muscle tissues (Figure 3.4). This pattern was evident for all species, including resident species *H. didactylus*, as well as for marine migrants and stragglers such as *D. labrax*, *S. aurata* or *S. solea*. This pattern could also be generally observed among the most frequently detected neuroactive pharmaceuticals, such as topiramate, venlafaxine and risperidone (Figure 3.4). Notwithstanding, not all species seem to accumulate neuroactive pharmaceuticals in the same range of concentrations, i.e., some were found to accumulate higher summed concentrations, such as *D. labrax* and *S. solea* (up to hundreds of ng/g), and to a less extent *P. flesus*, *H. didactylus* and *S. aurata* (up to tens of ng/g), whereas both *Diplodus* species showed reduced concentrations (up to 1.5 ng/g) (Figure 3.4). This may be the result of bioconcentration rates being influenced by different metabolic rates, linked to health status, feeding regimes, life-stage or size (Arnot and Gobas, 2006). Differences in bioaccumulation among wild fish species are known, and its association with species' ecological traits, including different habitat use, feeding strategies or trophic levels has been studied (e.g. Arnnok et al., 2017; Du et al., 2014; Fonseca et al., 2021; Huerta et al., 2018; Rojo et al., 2019). We hypothesised that estuarine resident species, that spend their

whole life cycle inside the estuary would have increased pharmaceutical accumulation compared to marine migrant or straggler species, which use the estuaries as nurseries or occasionally for feeding purposes, and thus spend comparatively less time inside the estuarine environment. Yet, our results show an unclear pattern in the bioaccumulation of different therapeutic groups across species with different habitat use classifications (Figure 3.5). This reveals how exposure to neuroactive compounds and consequent bioaccumulation in fish tissues does not imply exposure to contamination sources throughout their entire life or even large extended periods. Accordingly, it is known that pharmaceutical uptake and bioconcentration can occur in short timeframes (e.g. Liu et al., 2021; Wang and Gardinali, 2013), supporting the idea that all fish species tend to bioaccumulate neuroactive compounds, regardless of the time they spend in more prone areas inside the estuary. Notwithstanding, the specimens sampled here are late juveniles/young adults, which have most likely spent their first year(s) inside the estuary, which may contribute to the higher and comparable concentrations in marine migrants such as *D. labrax*, *S. solea and P. flesus* and those found in estuarine resident species *H. didactylus*.

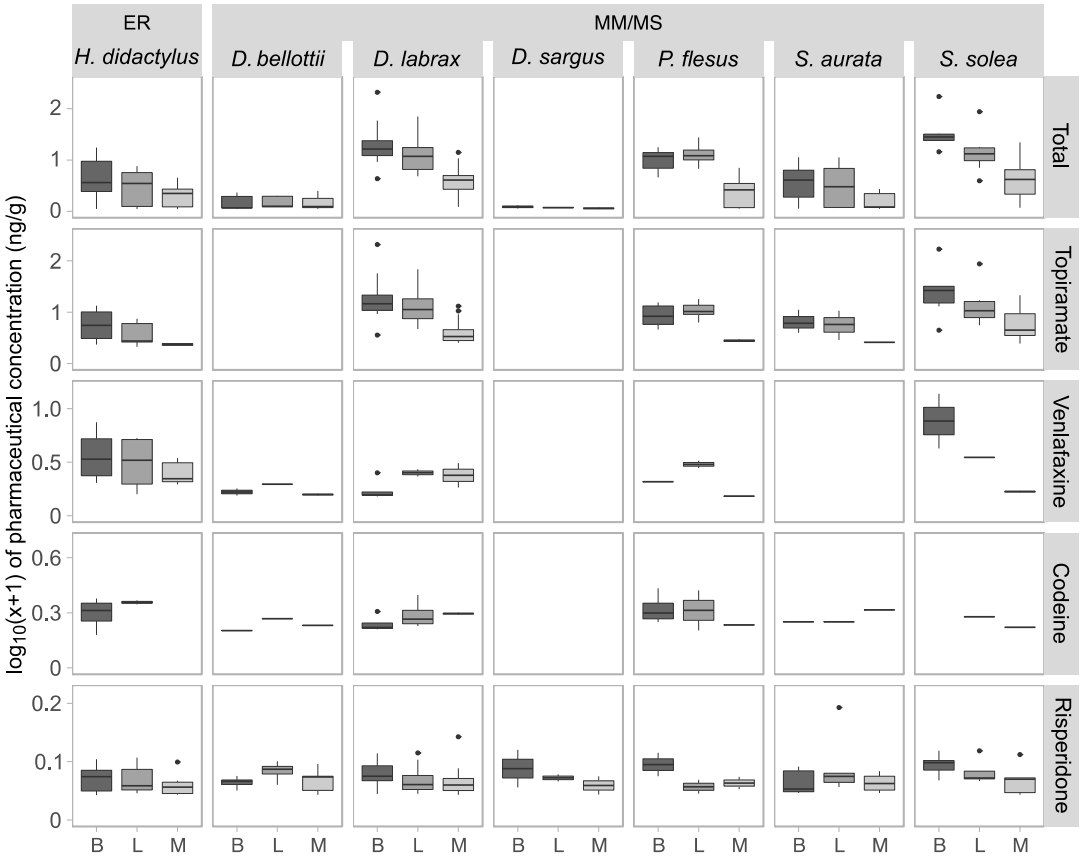

Figure 3.4. Concentrations of all neuroactive pharmaceuticals (Total) and of the most frequently detected pharmaceuticals (Topiramate, Venlafaxine, Codeine and Risperidone) in fish brain (B), liver (L) and muscle (M) in each fish species, namely estuarine resident (ER) *Halobatrachus didactylus* (N=14), and marine migrants and stragglers *Diplodus bellottii* (N=6), *Dicentrarchus labrax* (N=15), *Diplodus sargus* (N=2), *Platichthys flesus* (N=5), *Sparus aurata* (N=5) and *Solea solea* (N=8). Concentrations (ng/g ww) are presented as $\log_{10}(x+1)$, and scales differ between plots. Boxplots show median, 25th and 75th percentiles, upper and lower whiskers extending at most 1.5 times the interquartile range (IQR) to maximum and minimum values, respectively.

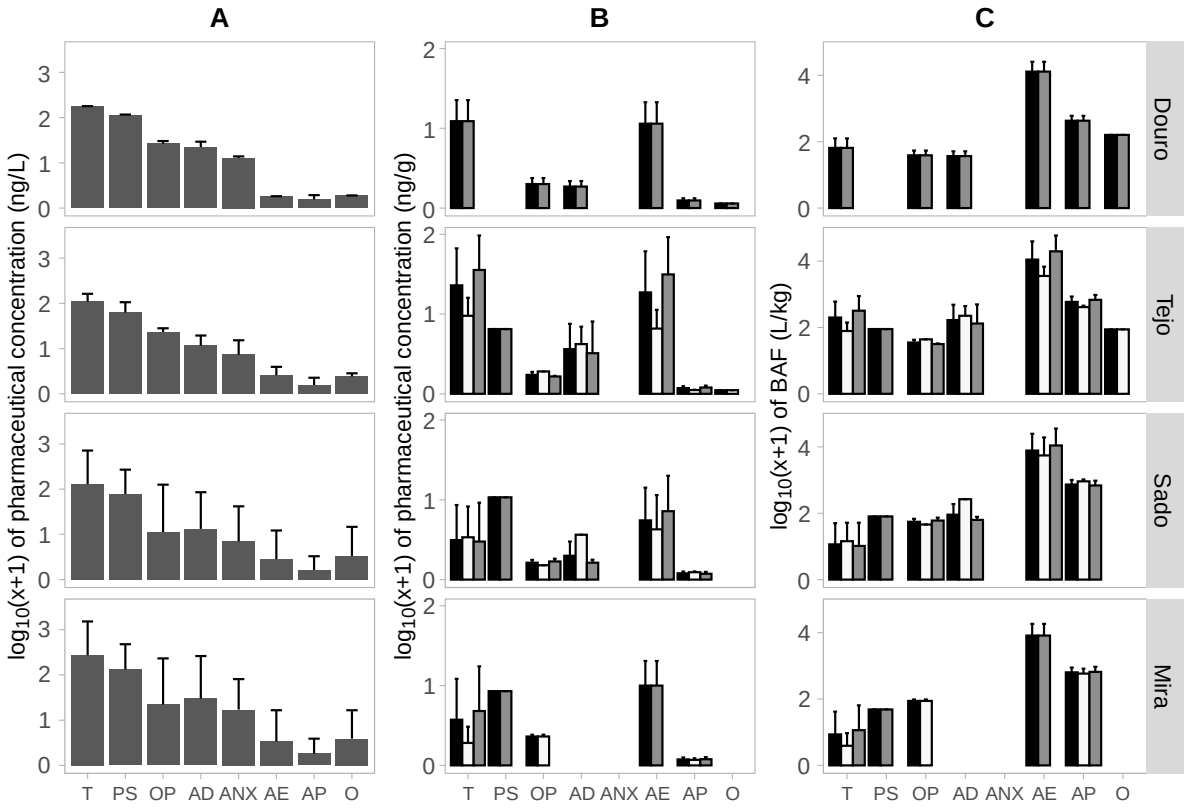

Figure 3.5. Mean (and standard deviation) of the summed concentrations per therapeutic class in the water (N=25) in ng/L (A) and fish brain (N=50, tissue concentrations in ng/g ww (B) and bioaccumulation factors in L/kg (C) are presented). Tissue concentrations and BAF from fish brain are given for all species (All, black bars), for estuarine resident species (ER, white bars) and marine migrant or straggler species (MM/MS, grey bars). Values are presented as $\log_{10}(x+1)$ and scales differ between plots. Therapeutic groups are the following: T - Total, PS - Psychostimulants, OP - Opioids, AD - Antidepressants, ANX - Anxiolytics, AE - Antiepileptics, AP - Antipsychotics and O - Other (including anticholinergic agents, hypnotics and sedatives, anti-dementia drugs).

Moreover, we addressed the potential link between bioaccumulation and fish trophic levels and found no significant correlations between total pharmaceutical concentrations (median values) and species trophic levels (TL, Table 3.2), for each of the three tissues, brain, liver and muscle ($r > -0.54$, p-value > 0.24). This points to a general bioaccumulation among all fish species, independently of trophic level, which is also highlighted by the overlap of data points obtained through the principal component analysis (Figure A2.2), showing that no specific pattern of bioaccumulation can be highlighted among species or estuaries.

Overall, the bioaccumulation of neuroactive compounds was observed for all seven fish species, in all four estuaries, with higher contributions from antiepileptics, psychostimulants, anxiolytics and antidepressants. A similar bioaccumulation pattern was generally evident among all species, revealing overall higher bioaccumulation in brain tissue, followed by liver and muscle, highlighting the importance of tissue selection in future bioaccumulation studies. No clear patterns were evident considering species' different habitat uses, including resident species, marine migrants or straggler species, and there was no obvious bioaccumulation pattern

in relation to the different trophic levels, indicating a general uptake of neuroactive pharmaceuticals among the seven fish species, despite higher summed concentrations could be found in some species.

Conclusion

This study analyses the occurrence of a broad suite of neuroactive pharmaceuticals of various therapeutic groups in estuarine surface waters and its bioaccumulation in three different tissues of seven species of fish with different life-history strategies and habitat use patterns. In the water, all seven therapeutic groups were frequently detected in all four estuaries (>78%) and almost half (15) of all neuroactive compounds exceeded concentrations of 10 ng/L, defined as the threshold level for studies on environmental fate and effects. With 10 and up to 26 neuroactive compounds detected in individual water samples, our results reveal a complex mixture of a suite of compounds in all four estuaries, despite differences in hydromorphology and urban development in the vicinity of the estuarine systems.

The bioaccumulation of neuroactive compounds was observed in all seven fish species collected in the different estuaries, with neuroactive compounds being detected in every fish brain and in 95% of fish liver and muscle tissues. A bioaccumulation pattern was evident among species, and in all estuaries, revealing overall higher bioaccumulation in the brain followed by liver and muscle tissues. Moreover, no clear uptake patterns linked to different habitat use or trophic levels were found, pointing to a conspicuous uptake of neuroactive pharmaceuticals among the different fish species.

Here, we reveal the ubiquity of neuroactive compounds in estuarine waters and the bioaccumulation of these compounds across multiple estuarine and marine fish species, independently of their estuary of capture, habitat use or trophic level. These results are key for improved risk assessment, yet information linking internalized concentrations to toxic effects is still scarce, though crucial for defining threshold safety levels to manage the risk of these compounds in the environment. Moreover, despite recent efforts concerning the impacts of pharmaceuticals in estuarine and marine environments, there is still a considerable knowledge gap regarding these key ecosystems when compared to freshwater systems, that needs to be addressed.

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CHAPTER 4

Biomarker and behavioural responses of an estuarine fish following acute exposure to fluoxetine

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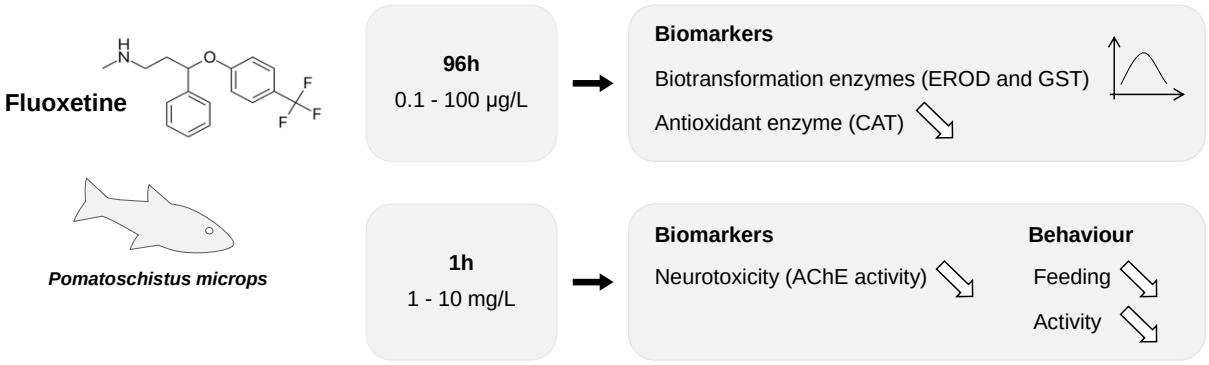

Abstract

Antidepressants such as fluoxetine are frequently detected in estuaries and can have profound effects on non-target organisms by interfering with the neural system and affecting essential physiological processes and behaviours. In this context, short-term effects of fluoxetine exposure were analysed in the common goby *Pomatoschistus microps*, an estuarine resident fish species. Two experiments were conducted with fish exposed to: i) fluoxetine concentrations within the µg/L range for 96h (0.1, 0.5, 10 and 100 µg/L) and ii) fluoxetine concentrations within the mg/L range for 1h (1, 5 and 10 mg/L). Acute toxicity was assessed via multiple biomarker responses, namely: activity levels of antioxidant (superoxide dismutase and catalase) and detoxification enzymes (ethoxyresorufin O-deethylase and glutathione S-transferase); and biomarkers of effects (lipid peroxidation and DNA damage) and of neurotoxicity (acetylcholinesterase inhibition). Furthermore, behavioural responses concerning activity (active time, movement delay and number of active individuals) and feeding (number of feeding individuals) were also recorded and analysed. Acute fluoxetine exposure for 96h (in the µg/L range) reduced antioxidant CAT activity with increasing concentrations but had no significant effect on SOD activity. Biotransformation enzymes showed bell-shaped response curves, suggesting efficient fluoxetine metabolism at concentrations up to 10 µg/L. No significant damage (LPO and DNAd) was observed at both concentration ranges (µg/L and mg/L), yet one hour exposure to higher fluoxetine concentrations (mg/L range) inhibited acetylcholinesterase activity (up to 37%). Fluoxetine (at mg/L) also decreased the number of both feeding and active individuals (by 67%), decreased fish active time (up to 93%) and increased movement delay almost 3-fold (274%). Overall, acutely exposed *P. microps* were able to cope with fluoxetine toxicity at the

µg/L range but higher concentrations (mg/L) affected fish cholinergic system and behavioural responses.

Keywords

Antidepressant, SSRI, Ecotoxicology, Estuaries, Fish, Biomarkers, Feeding, Behaviour

Introduction

Pharmaceuticals are continuously released to aquatic environments via multiple routes such as household, hospital and industrial wastewater effluents, aquaculture or animal husbandry, resulting in their ubiquitous presence in freshwater and coastal environments worldwide (Caldwell, 2016; Kümmerer, 2009). Consequently, a wide range of concentrations have been reported, usually within ng/L to µg/L range (Mezzelani et al., 2018), yet much higher concentrations, in the mg/L range, have also been reported in surface waters, chiefly associated with effluents from aquacultures and pharmaceutical manufacturing plants (Fick et al., 2009; Larsson et al., 2007; Le and Munekage, 2004). As pharmaceuticals are designed to produce effects at very low concentrations, their frequent detection in the aquatic environment raises concern over putative deleterious effects in non-target organisms.

Antidepressants and its metabolites have been frequently detected in the environment, with concentrations up to 1 µg/L in seawater, 8 µg/L in surface and groundwaters and up to 32 µg/L in wastewater treatment plants (Mezzelani et al., 2018). Among these are selective serotonin reuptake inhibitors (SSRIs), which are a group of pharmaceutical compounds used to treat depression and other psychiatric disorders. SSRIs act by blocking the reuptake of serotonin neurotransmitter from the synaptic cleft, increasing serotonin concentrations and consequently affecting neuronal signal transmission (Hiemke and Härtter, 2000). Allied to neuronal function, serotonin is also involved in other physiological mechanisms, such as those related to immune and endocrine systems or behavioural responses (Corcoran et al., 2010; Fent et al., 2006). Serotonin and its transporters are highly conserved in many species, particularly among vertebrates (Kreke and Dietrich, 2008; Mennigen et al., 2011), which implies SSRIs may elicit deleterious physiological and neuronal effects in a large number of species.

The antidepressant fluoxetine is one of the most prescribed SSRIs and is frequently detected in surface waters of estuarine and coastal areas (Mezzelani et al., 2018; Silva et al., 2012). Fluoxetine is considered highly toxic to various organisms (Corcoran et al., 2010; Fent et al., 2006), and even though there are inconsistencies across studies (Sumpter et al., 2014), detrimental effects of fluoxetine exposure have been observed in invertebrate and vertebrate species

(Sehonova et al., 2018; Silva et al., 2015), and at very short timeframes (i.e. within minutes to hours of exposure) (Ford and Fong, 2016). In fish, fluoxetine has been found to modulate gene transcription and enzymatic activities related to detoxification pathways, to alter endocrine and reproductive processes (e.g. reduce hormone production; fecundity and sexual development), as well as to alter behaviour (e.g. decreased feeding rates and locomotion) (e.g. Cunha et al., 2016; Giacomini et al., 2016; Henry and Black, 2008; Lister et al., 2009; Saaristo et al., 2017). Moreover, fluoxetine uptake and metabolism in fish is known to occur over a short timeframe (Paterson and Metcalfe, 2008) and it has been shown to accumulate in fish tissues (Brooks et al., 2005; Schultz et al., 2011). Yet, a considerable knowledge gap still exists concerning exposure effects on wildlife, particularly in marine and coastal environments (Gaw et al., 2014).

Biomarkers are sensitive measurements of biochemical, cellular or molecular interactions, that can signal early-on effects of exposure to xenobiotic compounds at the sub-individual level, and are therefore frequently used as indicators of exposure to and of effects in ecotoxicology studies (van der Oost et al., 2003). Recent studies have reported different effects of fluoxetine on biomarker responses in various aquatic organisms, albeit only a few evaluated *in vivo* fish exposures (e.g. Chen et al., 2018; Ding et al., 2016; Pan et al., 2018). At the individual level, behaviour is an ecologically relevant indicator of exposure to neuroactive compounds, as it may directly impact fitness and survival of aquatic organisms (Brodin et al., 2014).

In this context, the toxicity potential and effects of fluoxetine allied to its pervasive presence in the aquatic environment merits further exploration. Notably, analysing sub-lethal biological responses and behaviour changes of organisms exposed to a wide range of environmental concentrations of this neuroactive compound is of high ecological relevance, and should contribute to improve our understanding of its potential impact on estuarine biota. Accordingly, the aim of this study was to assess the effects of fluoxetine waterborne exposure on key biomarker and behavioural responses of *Pomatoschistus microps* (Krøyer, 1838), an estuarine resident fish species, pivotal to community functioning in temperate estuaries, and frequently used in ecotoxicology and biomonitoring studies (e.g. Fonseca et al., 2011; Oliveira et al., 2013). We conducted two independent short-term exposure experiments where fish were exposed to: i) fluoxetine concentrations within the µg/L range for 4 days (0.1, 0.5, 10 and 100 µg/L), covering the range of environmental concentrations reported for antidepressants and its metabolites; and ii) higher concentrations of fluoxetine for 1 hour (1, 5 and 10 mg/L), simulating acute exposure to point source contamination.

Accordingly, multiple biomarker responses were assessed in *P. microps*, namely: the activity levels of antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT),

responsible for protecting cells from reactive oxygen species (ROS) and thus for reducing oxidative stress; the activity of detoxification enzymes ethoxyresorufin O-deethylase (EROD) and glutathione S-transferase (GST), responsible for the metabolism of xenobiotic compounds, including pharmaceuticals; the levels of lipid peroxidation (LPO) and DNA damage (DNAd); and the activity of acetylcholinesterase (AChE) activity as an indicator of neurotoxicity. Concerning behavioural endpoints, we hypothesised that waterborne exposure to fluoxetine may alter locomotory and feeding behaviours in *P. microps*, and thus compromise individual fitness (e.g. by affecting fish ability to capture prey, avoid predatory attacks or to successfully reproduce), which would in the long-term reduce fish survival (Gerhardt, 2007). Ultimately, by combining sub-individual and individual responses, we intend to attain a more comprehensive assessment of fluoxetine toxicity on an estuarine fish species, a group which has seldom been evaluated.

Materials and methods

Fish sampling and acclimatization

P. microps individuals (length 3.01 ± 0.25 cm) were collected at low tide in the Tejo estuary natural reserve, near Alcochete (mean and standard deviation of water salinity and temperature were 19.2 ± 0.10 and 20.9 ± 0.26, respectively), using a hand net, and transported to the laboratory in a common tank (approx. 80L) with continuous aeration. Upon arrival, fish were divided randomly among three 80 L tanks, equipped with aeration and filtration systems. Throughout the day, a gradual shift to exposure water conditions was performed, with target values for temperature (ca. 20ºC) and salinity (18) similar to field water measurements. Fish were fed daily with newly hatched *Artemia* nauplii and worms (*Hediste diversicolor*). All procedures took place in a controlled temperature room, and water at 18 salinity was prepared with synthetic marine salt dissolved in filtered dechlorinated tap water.

Experimental design

Fish were allocated randomly among 15 experimental tanks, with 12 individuals per tank, and acclimated to exposure conditions for one week. The acute semi-static toxicity test was performed according to OECD guidelines (test no. 203) for 96 hours in 18L aerated glass tanks with natural photoperiod and no filtration. Four concentrations of the antidepressant fluoxetine and a control treatment were used (0, 0.1, 0.5, 10 and 100 µg/L), with three replicate tanks per concentration. Concentrations used in this trial cover the range of reported environmental concentrations for antidepressants and its metabolites (Mezzelani et al., 2018).

Fluoxetine stock solutions were prepared with milliQ-grade water and stored at -20ºC. Daily water renewals were performed, and fluoxetine concentrations appropriately restored to maintain fluoxetine concentrations in the tanks. Water parameters, namely temperature, salinity, pH and ammonia, as well as fish mortalities were recorded daily. Feeding was suspended 24h before the beginning of the exposure test.

After 96h exposure, fish were transferred to individual behavioural observation tanks and rested for 10 minutes in the new environment before each trial, to avoid handling stress interference. All tanks were covered throughout the experimental trials and observations were made through recorded high-definition video, to minimize any potential stress or bias caused by visual contact/human presence. In feeding trials, 10 *Artemia* nauplii were released per tank, marking the beginning of a 5-minute observation period for feeding and locomotory activities. Analysed behavioural endpoints included the percentage of active and feeding individuals, the overall time individual fish spent moving and the time individual fish took to make the first movement (i.e. movement delay). After behavioural trials, fish were immediately sacrificed, and tissues stored at -80ºC until further analysis.

Fluoxetine uptake and metabolism in fish is known to occur over a short timeframe, within 5h of exposure to low concentrations (0.55 µg/L) (Paterson and Metcalfe, 2008). Hence, we hypothesized that 1 hour of exposure to a higher range of concentrations (mg/L) would allow for fluoxetine uptake and metabolism and would suffice to generate biological or behavioural effects in *P. microps*. Accordingly, an acute static toxicity test was conducted, where fish were individually exposed to three fluoxetine concentrations and a control treatment (0, 1, 5 and 10 mg/L) for 1h, in 1L glass beakers with water also at 20 ºC and 18 salinity. Concentrations used in this trial were chosen to mimic acute exposure to point source contamination (Fick et al., 2009; Larsson et al., 2007). Twelve fish were exposed per treatment and post-exposure procedures were performed as described above.

All experimental procedures were performed in accordance with animal testing guidelines and licenced by university animal welfare committee and national authorities.

Biomarkers quantification

For biomarkers' quantification different fish tissues were dissected, namely liver, head and gills. Tissue samples were homogenized in cold 100 mM monobasic potassium phosphate/dibasic potassium phosphate (K_2HPO_4/KH_2PO_4) buffer (pH 7.4) containing 0.15 M KCl (potassium chloride), 0.1 mM PMSF (phenylmethylsulfonyl fluoride), 1 mM DTT (dithiothreitol) and 1 mM EDTA (ethylenediaminetetraacetic acid) to avoid protein degradation. Four

individuals were pooled for liver samples and a 1:5 (w/v) tissue:buffer ratio was used in homogenization, whilst head and gills were individually homogenized in 1 and 0.5 mL of the same buffer at pH 7.2, respectively.

Aliquots of liver homogenate were separated for lipid peroxidation (LPO), to which BHT (butylated hydroxytoluene) at 4% was added (1:15 v/v sample) to prevent further lipid peroxidation. The remaining liver homogenate was centrifuged at 12000 g for 20 min at 4°C, and aliquoted for superoxide dismutase (SOD), catalase (CAT), ethoxyresorufin-*O*-deethylase (EROD) and glutathione *S*-transferase (GST) determination. Gills homogenates were aliquoted for DNA damage quantification, while head homogenates were centrifuged at 11000 g for 3 min at 4°C and used in acetylcholinesterase (AChE) activity analysis.

All biomarker responses were determined in 96-well microplates and each reading was done in triplicate, using a microplate reader (Biotek Synergy HT). Protein content was adjusted to 0.5 – 0.7 mg mL^{-1} for biomarker determinations, except for AChE assays, for which protein content was adjusted to 0.3 mg mL^{-1}.

Superoxide dismutase (SOD) was measured according to Marklund and Marklund (1974), based on its ability to inhibit pyrogallol autoxidation, with few adaptations. Briefly, increase in absorbance was followed for 5 min at 325 nm, after incubation of 5 µL of homogenate with 265 µL of 50 mM phosphate buffer (pH 7.8) containing 1 mM EDTA, and 30 µL of a 30 mM pyrogallol solution in 10 mM HCl. Control assays were performed in the absence of homogenate samples to determine pyrogallol autoxidation. SOD activity was expressed as U mg^{-1} of total protein concentration, with one unit of SOD as the amount of enzyme that inhibits the reduction of pyrogallol by 50%.

Catalase (CAT) activity was determined according to Aebi (1974), by measuring the decrease in absorbance at 240 nm, caused by substrate consumption. Briefly, 130 µL of 50 mM phosphate buffer were added to 20 µL of sample, and the reaction was started with the addition of 150 µL of substrate (30 mM H_2O_2 in 50 mM phosphate buffer, pH 7). CAT activity was then calculated as the difference in absorbance per unit of time ($\varepsilon = -0.04$ mM^{-1} cm^{-1}) and expressed as µmol per minute per mg of total protein concentration.

Ethoxyresorufin-*O*-deethylase (EROD) activity was determined following Burke and Mayer (1974) method, with few adaptations by Fernandes et al. (2002). The reaction was initiated with the addition of 10 µL of NADPH (8.33 mg mL^{-1}) to 190 µL of 7-ethoxyresorufin solution (0.1 mg mL^{-1} in 100 mM phosphate buffer (pH 7.4)) and 100 µL of sample, at 30 °C for 20 min. Fluorescence from 7-hydroxyresorufin was measured at 537/583 nm excitation/emission wavelengths, and resorufin sodium salt was used as standard. Activity was

calculated as the amount of resorufin (ρmol) generated per mg of total protein per minute of reaction time.

Glutathione S-transferase (GST) activity was determined following Habig et al. (1974). Briefly, the conjugation of glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) by GST was measured through changes in absorbance at 340 nm ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$), for 3 min. The assay was started with the addition of 250 µL of a final reaction mixture containing 100 mM phosphate buffer (pH 6.5), 20 mM CDNB and 20 mM reduced glutathione, to 50 µL of sample. GST activity was expressed as nmol CDNB conjugate formed per mg of total protein per minute of reaction.

Lipid peroxidation (LPO) was determined according to Ohkawa et al. (1979). The reaction of thiobarbituric acid reactive substances (TBARS) with 2-thiobarbituric acid (TBA) occurred after incubation of 500 µL of TCA 12%, 450 µL of 60 mM Tris-HCl (pH 7.4) containing 0.1 mM EDTA and 500 µL of TBA 0.73% with 50 µL of sample for 60 min, at 97 °C. Samples were cooled on ice and centrifuged at 13400g for 3 min, and absorbance was measured at 535 nm ($\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$). LPO was expressed as nmol of TBARS formed per mg of total protein.

DNA damage (DNAd) was determined in gills following the DNA alkaline precipitation assay by Olive (1988). Samples (50 µL) were first mixed with 250 µL of a 2% SDS solution containing 10 mM EDTA, 10 mM Trisbase (pH 12.4) and 50 mM NaOH. Then, 250 µL of a 0.12 M KCl solution were added and the mixture was incubated at 60 °C for 10 min. After cooling down on ice for 15 min, the mixture was centrifuged at 8000g for 5 min, at 4 °C. Following the addition of 200 µL of Hoechst dye (1 $\mu g\ mL^{-1}$ in 0.1 M K-phosphate buffer, pH 7.4) to 50 µL of the mixture, DNA concentration in the supernatant was determined at 360 nm/460 nm of excitation/emission wavelengths. Fluorescence values were compared to a DNA standard curve and DNAd was expressed as µg DNA per mg of total protein.

P. microps' head homogenates (cleaned of gills) were used for determination of acetylcholinesterase (AChE) activity, which has been described as the main cholinesterase form in this species' head tissues, and a proxy of brain AChE (Monteiro et al., 2005). Acetylcholinesterase (AChE) was determined according to Ellman et al. (1961), adapted to microplate (Guilhermino et al., 1996). Briefly, 250 µL of a final reaction mixture containing 100 mM phosphate buffer (pH 7.2), 75 mM acetylthiocholine and 10 mM DTNB (5,5'-dithio-bis(2-nitrobenzoic acid)) were added to 50 µL of sample (protein adjusted to 0.3 $mg\ mL^{-1}$). The reaction of thiocholine with DTNB to produce the yellow anion TNB, was followed at 412 nm ($\varepsilon = 13.6\ mM^{-}$

^{1} cm^{-1}), every 20 secs for 10 min. The enzymatic activity was expressed in nmol of substrate hydrolysed per minute per mg of total protein.

Protein content was quantified following Bradford's method, adapted to microplate: 250 µL of Sigma Bradford solution is added to each replicate of sample (10 µL) and incubated for a 15 min period (light protected and at room temperature) after which absorbance is measured at 595 nm. Bovine serum albumin solution (1 mg mL^{-1}) was used as protein standard.

Data analyses

Data was first checked for normality and homogeneity of variances and transformed when necessary in order to meet these assumptions (using Shapiro-Wilk and Levene tests, respectively). In the 96h experiment, differences in biomarker responses among replicate tanks per treatment (n = 3) were first tested through analysis of variance. No differences were found among replicate tanks, except for one control tank in one biomarker response (DNAd). Since statistical results did not differ for DNAd analysis when considering tank and individual responses, for consistency with other biomarker responses, we present DNAd results based on all measurements in the following analyses. Accordingly, differences in biomarker responses among treatments in both experiments were tested through analyses of variance (ANOVA), followed by post-hoc Tukey tests. Number of replicates per treatment in the 96h experiment were n = 9 for SOD, CAT, EROD, GST and LPO and n = 12 for DNAd and AChE; whilst in 1h experiment the number of replicates were n = 3 for SOD, CAT, EROD, GST and LPO, n = 4 for DNAd and n = 6 for AChE. To test for independence of behavioural responses to treatment we used Kruskal-Wallis test (active time and movement delay, n = 12) and Fisher's exact test of independence (number of active and feeding individuals, n = 12), followed by post-hoc tests. According to data normality assumptions, Pearson product moment correlation (r_p) analysis was used to test for correlations among biomarker responses (parametric data), and Spearman rank correlation coefficients (r_s) to test for correlations between biomarkers and behavioural responses (non-parametric data). All analyses were performed in R software (RStudio Team, 2016), and a significance level of 0.05 was considered for all statistical tests used.

Results

Water parameters were constant across tanks and exposure days. Temperature (20.7°C ± 0.2°C), salinity (18.1 ± 0.1), pH (7.3 ± 0.2), and conductivity (26.8 uS ± 0.2 uS) were measured daily, and ammonia levels were maintained under 0.2 mg/L. Three individuals from three

different tanks died over the 96h experiment (one in the control, one in the 10 µg/L and one in the 100 µg/L tank), thus mortality did not exceed 10%, as recommended by OECD guidelines.

Following 96h of exposure to fluoxetine concentrations within the µg/L range (0.1, 0.5, 10 and 100 µg/L), dose-dependent inhibition of catalase activity with fluoxetine was observed, with significant differences from the control group at 10 and 100 µg/L ($F = 3.95$, p-value < 0.01, Fig. 4.1b). Concerning biotransformation enzymes, bell-shaped response curves were observed for both GST and EROD activity ($F > 4.8$, p-value < 0.01, Fig. 4.1c and d). No significant effects were observed in SOD activity, in LPO and DNAd levels, or in AChE activities ($F > 0.36$, p-value > 0.05, Fig. 4.1a, e, f and g). Concerning behaviour, no significant effects of fluoxetine were found after 96h ($\chi^2 > 11.77$, Fig. 4.2a and b; $H > 2.80$, Fig. 4.2c and d; p-values > 0.05). Few correlations were found among *P. microps* responses in the 96h experiment. Specifically, GST activity was positively correlated with EROD ($r_p = 0.64$, p-value < 0.001) and SOD ($r_p = 0.60$, p-value < 0.001) activities. EROD activity was positively correlated to SOD activity ($r_p = 0.38$, p-value < 0.01) and negatively to LPO levels ($r_p = -0.31$, p-value < 0.05).

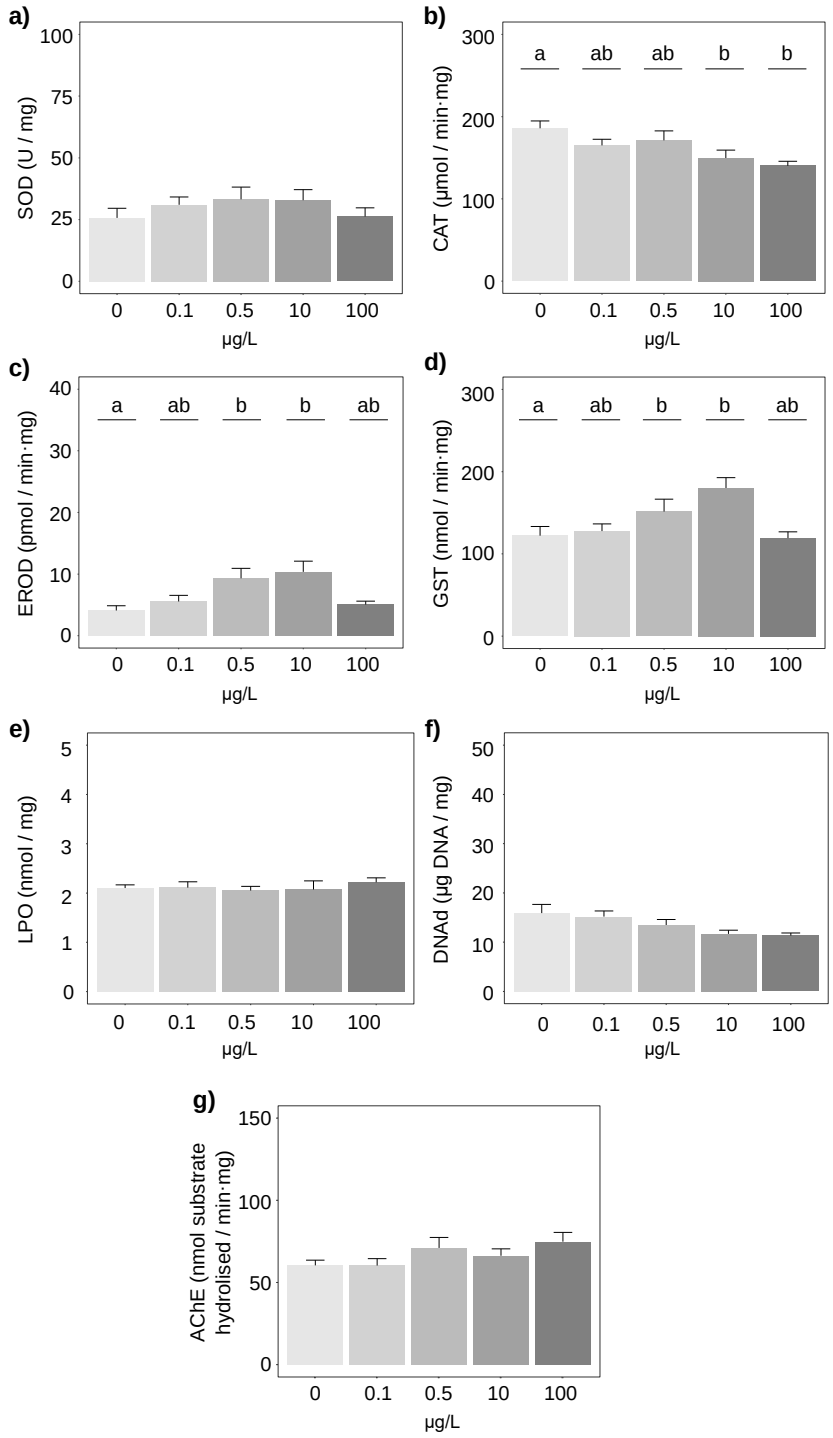

Figure 4.1. Biomarker responses of *P. microps* exposed to fluoxetine (µg/L) for 96h. One control and four fluoxetine treatments were tested (0, 0.1, 0.5, 10 and 100 µg/L). Bar plots with mean and standard deviations of biomarkers responses: a) SOD (superoxide dismutase) activity, b) CAT (catalase) activity, c) EROD (ethoxyresorufin O-deethylase) activity, d) GST (glutathione S-transferase) activity, e) LPO (lipid peroxidation) levels, f) DNAd (DNA damage) and g) AChE (acetylcholinesterase) activity. Different letters indicate significant differences from post-hoc comparison Tukey tests, following a one-way analysis of variance for each biomarker response. Number of replicates per treatment: n = 9 for SOD, CAT, EROD, GST and LPO; n = 12 for DNAd and AChE.

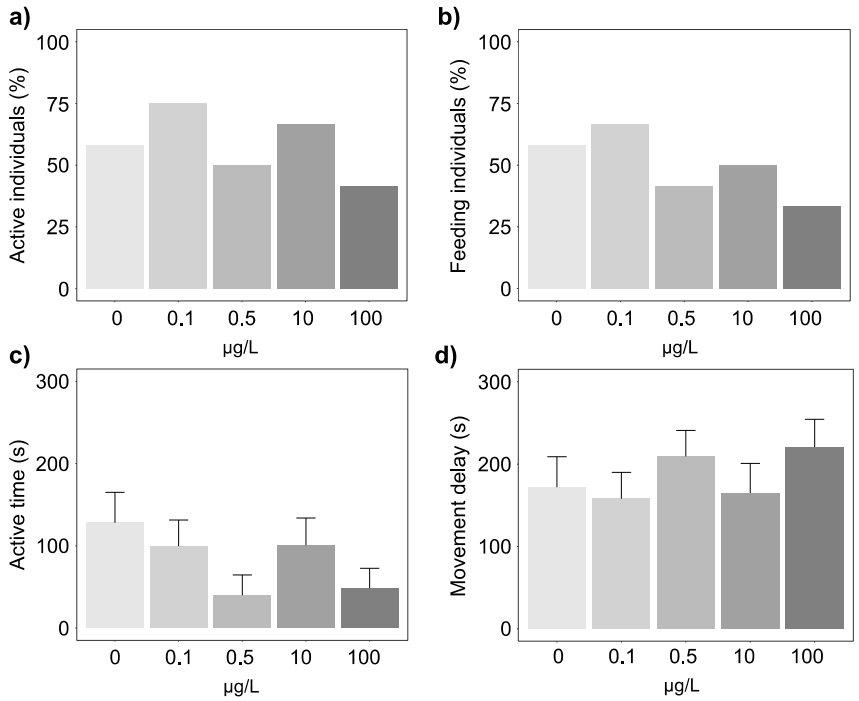

Figure 4.2. Behavioural responses of *P. microps* exposed to fluoxetine (µg/L) for 96h. One control and four fluoxetine treatments were tested (0, 0.1, 0.5, 10 and 100 µg/L). Bar plots of the percentage of a) active individuals and b) feeding individuals, and of mean and standard deviations of c) fish active time and d) movement delay. Different letters indicate significant differences from post-hoc analysis, following Fisher's exact test of independence (number of active and feeding individuals) and Kruskal-Wallis test (active time and movement delay), with n = 12 replicates per treatment for each behaviour endpoint.

After 1 hour of exposure to 1, 5 and 10 mg/L concentrations of fluoxetine, AChE activity was significantly inhibited by 27 and 37%, at 5 and 10 µg/L, respectively ($F = 5.60$, p-value < 0.01, Fig. 4.3g). For CAT and LPO, differences among treatments but not to control were observed ($F > 4.80$, p-value < 0.05, Fig. 4.3b and e). However, no significant changes were observed in SOD, EROD and GST enzymes activities nor in DNAd ($F > 0.05$, p-value > 0.05, Fig. 4.3a, c, d and f). Yet, fluoxetine at the highest concentration (10 mg/L) significantly reduced the number of active and feeding individuals, both by 67% ($\chi^2 > 11.77$, p-value < 0.01, Fig. 4.4a and b). Moreover, the active time of individual fish significantly decreased with exposure to fluoxetine at all concentrations tested ($H = 19$, p-value < 0.001, Fig. 4.4c). This decrease was concentration-dependent and ranged from 55% at the lowest concentration (1 mg/L) up to 93% at the highest concentration (10 mg/L). Furthermore, significant delays in fish movement were observed after exposure to fluoxetine at all concentrations tested ($H = 16.11$, p-value < 0.001, Fig. 4.4d), increasing from 69 seconds on average in control to 173 seconds at 1 and 5 mg/L (152 and 153%, respectively) and to 256 seconds on average (274%) at 10 mg/L exposure treatment. Correlations among *P. microps* responses were also observed in the 1h experiment. GST was positively correlated with EROD ($r = 0.62$, p-value < 0.05), and negatively with fish active time ($r_s = -0.60$, p-value < 0.05). AChE activity was positively correlated with fish active time

(r_s = 0.74, p-value < 0.001) and negatively with fish movement delay (r_s = -0.53, p-value < 0.01).

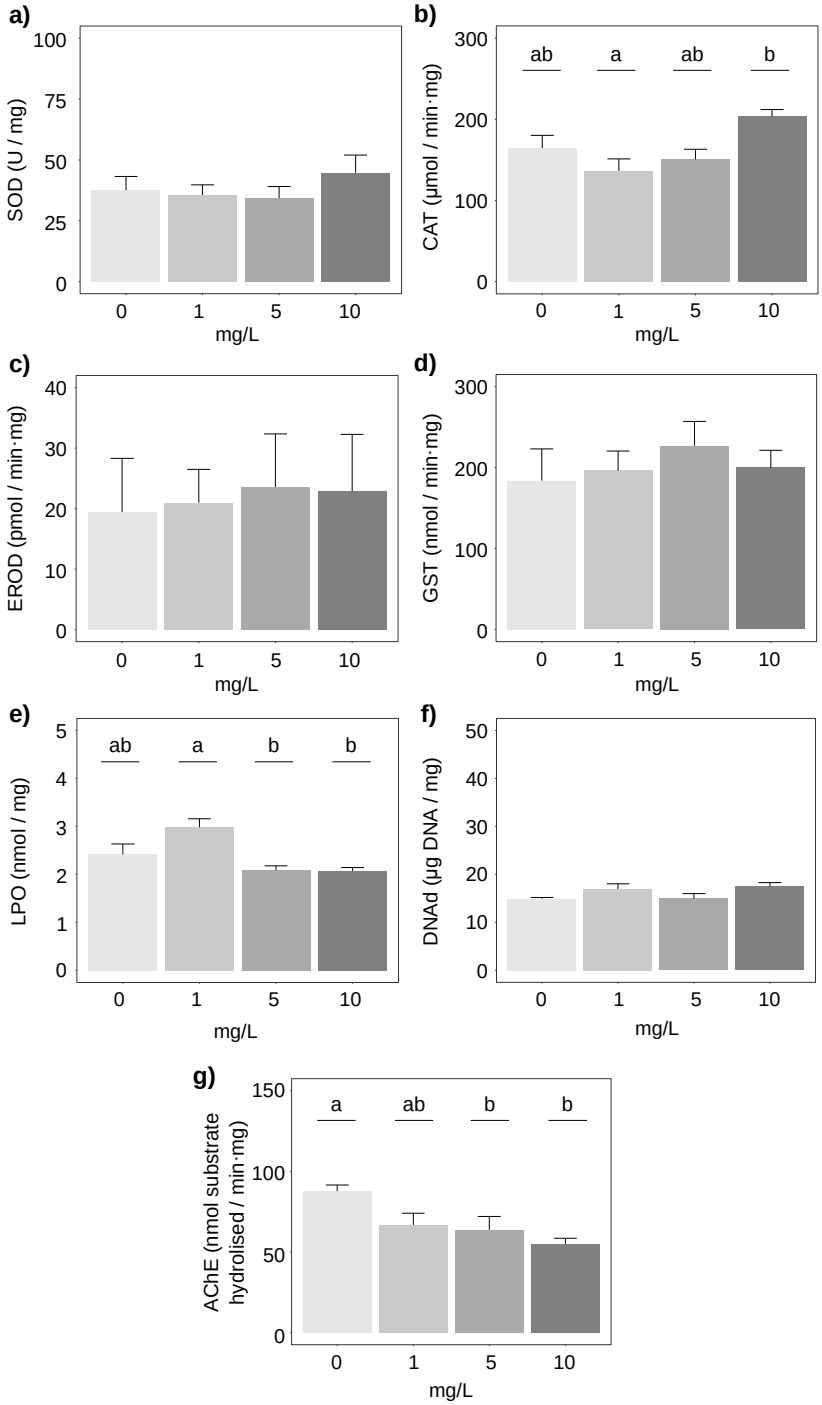

Figure 4.3. Biomarker responses of *P. microps* exposed to fluoxetine (mg/L) for 1h. One control and three fluoxetine treatments were tested (0, 1, 5 and 10 mg/L). Bar plots with mean and standard deviations of biomarkers responses: **a)** SOD (superoxide dismutase) activity, **b)** CAT (catalase) activity, **c)** EROD (ethoxyresorufin O-deethylase) activity, **d)** GST (glutathione S-transferase) activity, **e)** LPO (lipid peroxidation) levels, **f)** DNAd (DNA damage) and **g)** AChE (acetylcholinesterase) activity. Different letters indicate significant differences, from post-hoc comparison Tukey tests, following a one-way analysis of variance for each biomarker response. Number of replicates per treatment: n = 3 for SOD, CAT, EROD, GST and LPO; n = 4 for DNAd and n = 6 for AChE.

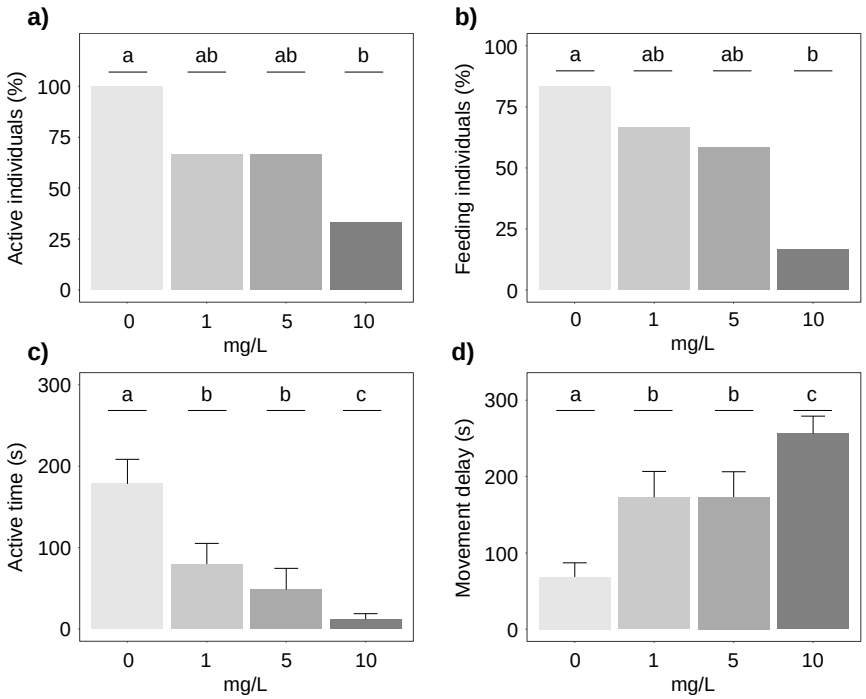

Figure 4.4. Behavioural responses of *P. microps* exposed to fluoxetine (mg/L) for 1h. One control and three fluoxetine treatments were tested (0, 1, 5 and 10 mg/L). Bar plots of the percentage of a) active individuals and b) feeding individuals, and of mean and standard deviations of c) fish active time and d) movement delay. Different letters indicate significant differences from post-hoc analysis, following Fisher's exact test of independence (number of active and feeding individuals) and Kruskal-Wallis test (active time and movement delay), with n = 12 replicates per treatment for each behaviour endpoint.

Discussion

Acute exposure to fluoxetine altered several biomarker responses in *P. microps*, although responses differed between the 96h exposure trial (from 0.1 to 100 µg/L concentrations) and the 1h exposure trial at higher concentrations (from 1 to 10 mg/L). Fish behavioural changes (feeding and locomotor activity) and neurotoxicity (acetylcholinesterase activity) were only observed after 1h exposure to higher concentrations (mg/L).

Biotransformation enzymes are responsible for the metabolism of different xenobiotic compounds, including pharmaceuticals. Induction of biotransformation enzymes following fluoxetine exposure has been reported *in vitro* (Thibaut and Porte, 2008) and *in vivo* in fish (e.g. Chen et al., 2018). However, fluoxetine, and its metabolite norfluoxetine, have been found to accumulate in fish tissues (Brooks et al., 2005; Paterson and Metcalfe, 2008), as well as to inhibit different CYP isoforms at high concentrations (mg/L range) (Smith et al., 2012; Thibaut et al., 2006), including EROD (Laville et al., 2004). In this study, positive correlations between biotransformation enzymes in both exposure trials points to fluoxetine metabolism in *P. microps'* liver, although significant differences were only evident in the 96h trial. Fluoxetine modelled EROD and GST activities the same way (in the µg/L range), with increasing enzymatic

activity up to 10 µg/L then returning to basal levels at higher concentrations (100 µg/L). This follows the hormetic model, which describes low-dosage induction of enzymatic activity followed by inhibition at higher dosages, resulting in a bell-shaped response curve (Calabrese and Baldwin, 2003). The reduction in activity of biotransformation enzymes at higher concentrations can result from downregulation of genes involved in detoxification pathways, as observed by Cunha et al. (2016), or from direct enzyme inhibition by fluoxetine and/or its metabolites.

Antioxidant enzymes, such as SOD and CAT, are the primary defence mechanisms against reactive oxygen species (ROS), which may be a product of chemicals exposure and uptake (van der Oost, 2003). Fluoxetine cytotoxicity at high concentrations (mg/L) has been linked to increased ROS production in fish hepatocyte cells (Laville et al., 2004), and only a few studies have explored fluoxetine effects on antioxidant enzymes' activity in fish *in vivo*, yet with varying responses. For instance, Pan et al. (2018) found total antioxidant capacity (T-AOC), and CAT and SOD activities significantly increased after 3 day exposure to fluoxetine at 0.1 µg/L in the goldfish (*Carassius auratus*). On the other hand, Ding et al. (2016) reported that a 7 day exposure to higher concentrations of fluoxetine (4 to 100 µg/L) caused a significant reduction in SOD activity in the same species. Cunha et al. (2016) also reported SOD inhibition, yet increased CAT activity in zebrafish embryos exposed to fluoxetine (0.4 to 247.5 µg/L) for 80h. In this study, catalase activity was the only biomarker to vary in both exposure trials, with a decreasing trend in activity with increasing fluoxetine concentrations after 96h at low concentrations (µg/L). On the other hand, no significant fluoxetine effects were observed in SOD activity. Contrary to previous findings, our results suggest that acute exposure to both fluoxetine concentration ranges (µg/L and mg/L) does not generate overt oxidative stress in *P. microps*, which is further supported by the lack of oxidative damage in lipids and DNA. LPO levels and DNA damage showed no significant alterations in comparison to control treatments, even at higher concentrations (mg/L). Yet, Ding et al., (2016) and Chen et al., (2018) have previously reported increased levels of lipid peroxidation in fish exposed to fluoxetine at µg/L concentrations after 7 and 42 days exposure, respectively. To the best of our knowledge, this is the first study to assess DNA damage in fish exposed to fluoxetine, yet *in vitro* studies with invertebrate species have shown fluoxetine genotoxicity and DNA damage at concentrations as low as ng/L (e.g. Gagné et al., 2006; Lacaze et al., 2015), whilst others have reported decreased or no DNAd *in vivo* (e.g. Franzellitti et al., 2015; Magni et al., 2017; Maranho et al., 2014).

Differences in antioxidant responses among studies may be related to different experimental settings, such as exposure time and concentrations tested, as well as to different life-stages and species-specific responses. Smith et al. (2010) described high intra-species

variability in *in vitro* hepatic fluoxetine metabolism in four fish species, which hindered inter-species comparisons. Noteworthy, in *P. microps,* the reduced antioxidant responses we observed following acute fluoxetine exposure could result from expedite fluoxetine biotransformation and excretion, which would minimize antioxidative response and prevent oxidative damage in this species. Alternatively, other antioxidant mechanisms not measured in this study could be contributing to low oxidative stress levels. Furthermore, at the mg/L range, an increasing trend in antioxidant enzymes with increasing concentrations could be observed, with consequent reduction of LPO levels. Although Paterson and Metcalfe (2008) found rapid uptake and metabolism of fluoxetine in fish within 5 hours of exposure to 0.55 µg/L, our results suggest that the activation of antioxidant defences in *P. microps* may require exposure periods longer than 1 hour or much higher concentrations (5 or 10 mg/L). In this context, studies of short-term (hourly) exposures to enhanced concentrations of pharmaceuticals in biota are paramount to screen for affected mechanisms (e.g. Hamilton et al., 2016; Magno et al., 2015). Furthermore, identification of fluoxetine metabolic pathways, biotransformation efficiency and tissue bioaccumulation across fish species warrants further investigation.

Only recently has AChE activity been measured in fish brains and shown to increase at concentrations ranging from 0.1 to 200 µg/L in acute and chronic exposures (Chen et al., 2018; Pan et al., 2018). In this study, no significant effects were observed in *P. microps* AChE activity in the 96h (exposure in µg/L) trial, although an increasing trend in activities could be observed, and is in line with the previous studies. However, in the 1h exposure trial (exposure in mg/L), AChE activity was significantly inhibited at 5 and 10 mg/L concentrations. In human serum, cholinesterase inhibition also occurred at high fluoxetine concentrations, in the mg/L range (ca. 0.9 to 18 mg/L) (Müller et al., 2002). Fluoxetine and other SSRIs also evidenced a dose-dependent inhibitory effect on zebrafish embryos cholinesterases (Farias et al 2019; Yang et al., 2018). Accordingly, high fluoxetine concentrations and rapid uptake and accumulation in fish brain (Paterson and Metcalfe, 2008; Schultz et al., 2011) likely lead to the prompt AChE inhibition even after only one hour of exposure.

Decreased locomotor activity and latency in movement have been described in different fish species after short and long-term exposures to fluoxetine at both ng/L and µg/L range (e.g. Meijide et al., 2018; Saaristo et al., 2018; Winder et al., 2012). Fluoxetine has also been associated to decreased feeding rates in fish at these concentration ranges (e.g. Mennigen et al., 2010; Weinberger and Klaper, 2014), which could be linked to either reduced appetite (due to serotonin modulation) or indirect effects on activity (e.g. reduced locomotion and stimuli response) (McDonald, 2017). At higher concentrations, in the mg/L range, hourly exposures have

induced changes in behaviour in fish and invertebrates (Hamilton et al., 2016; Magno et al., 2015). In our study, only exposure to higher fluoxetine concentrations (mg/L over an hour) caused adverse effects on feeding and activity patterns of *P. microps*. Specifically, individual fish were less active and movement delay was increased at all concentrations in the mg/L range. The number of active and feeding individuals was also significantly reduced at 10 mg/L. However, *P. microps* behaviour was not affected after 96h exposure to concentrations of up to 100 µg/L, suggesting that this species behavioural responses were less sensitive to fluoxetine exposure in comparison to previous studies. Typically, estuarine species are well adapted to the high natural variability of these environments, which allows them to tolerate stressful conditions, including of anthropogenic origin (Elliott and Quintino, 2007). Albeit fish used in this study were collected at a natural reserve site, we cannot exclude prior exposure (and consequent conditioning) of fish to fluoxetine and other SSRIs, which have been identified in the area, although at very low concentrations (< 10 ng/L, Reis-Santos et al., 2018). Furthermore, the majority of previous studies were performed in freshwater fish species and laboratory reared individuals (e.g. zebrafish, minnow, goldfish), thus different responses to fluoxetine toxicity could be linked to inter-species evolutionary differences. Brown et al. (2014) highlighted differential susceptibility of fish to pharmaceuticals, based on evolutionary divergence in species drug-target activation, physiology, behaviour and ecology.

Given fluoxetine's mode of action, behavioural changes have been associated with modulation of the serotonergic system. The strong correlations between *P. microps* AChE activity and fish movement delay (negative correlation) and active time (positive correlation) further suggests that changes in fish activity could also be linked to alterations in the cholinergic system. This hypothesis has also been suggested by other authors as a possible route of behaviour modulation (e.g. Farias et al., 2019; Winder et al., 2012). Therefore, AChE activity could be a suitable biomarker of fluoxetine toxicity in behavioural studies, yet links between serotonergic and cholinergic pathways and fish behaviour need to be further resolved. A metabolomics approach could provide valuable insights into metabolic pathways and interactions between these two systems and behaviour responses following exposure to SSRIs.

In conclusion, acute exposure to fluoxetine induced hepatic biotransformation enzymes in *P. microps*, yet no significant oxidative stress responses were observed. Behavioural and neurotoxic effects were only observed at higher concentrations (mg/L). Nonetheless, further insights into the variability of inter-specific responses, as well as into chronic exposure effects at environmental relevant concentrations in non-model species, are needed to improve environmental risk assessment of fluoxetine and other SSRIs.

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CHAPTER 5

Depressed, hypertense and sore: Long-term effects of fluoxetine, propranolol and diclofenac exposure in a top predator fish

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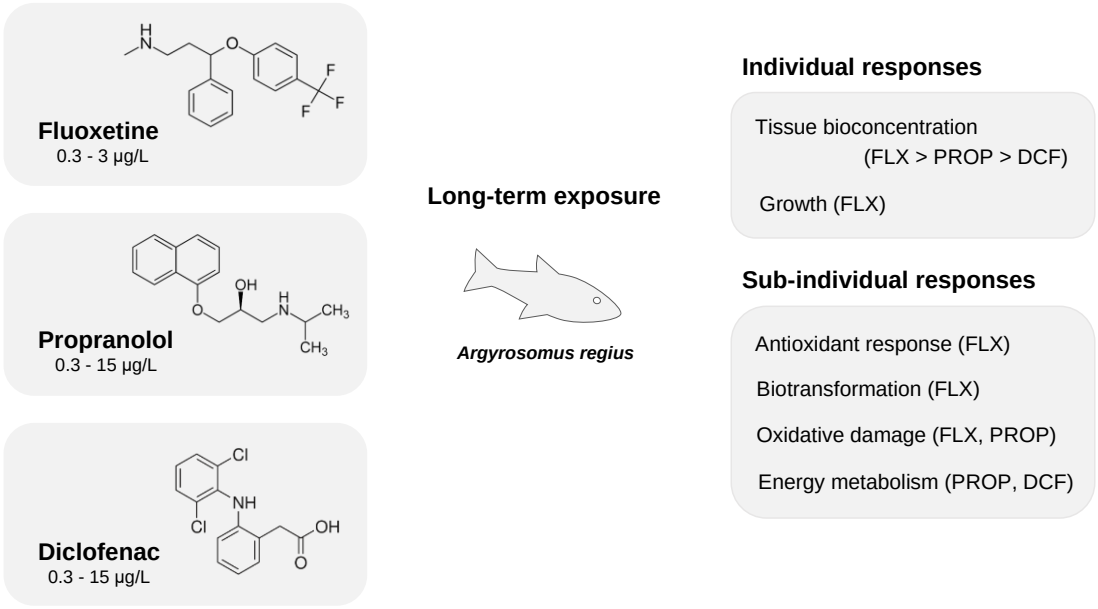

Abstract

Pharmaceutical compounds are continuously released into the aquatic environment, resulting in their ubiquitous presence in many estuarine and coastal systems. As pharmaceuticals are designed to produce effects at very low concentrations and target specific evolutionary conserved pathways, there are growing concerns over their potential deleterious effects to the environment and specifically to aquatic organisms, namely in early life-stages. In this context, the long-term effects of exposure of juvenile meagre *Argyrosomus regius* to three different pharmaceuticals were investigated. Fish were exposed to environmental concentrations of one of three major used pharmaceuticals: the antidepressant fluoxetine (0.3 and 3 µg/L for 15 days), the anti-hypertensive propranolol and the non-steroidal anti-inflammatory agent diclofenac (0.3 and 15 µg/L for 30 days). Pharmaceuticals bioconcentration in fish muscle was examined, along with biomarkers in different tissues related with antioxidant and biotransformation responses (catalase, superoxide dismutase, ethoxyresorufin-*O*-deethylase and glutathione *S*-transferase), energetic metabolism (lactate dehydrogenase, isocitrate dehydrogenase and electron transport system activities), neurotransmission (acetylcholinesterase activity) and oxidative damage (DNA damage and lipid peroxidation levels). Overall, each pharmaceutical had different potential for bioconcentration in the muscle (FLX > PROP > DCF) and induced different

biological responses: fluoxetine was the most toxic compound to juvenile meagre, affecting fish growth, triggering antioxidant defense responses, inhibiting detoxification mechanisms and increasing lipid peroxidation and DNA damage in the liver; propranolol exposure increased DNA damage and decreased aerobic metabolism in fish muscle; and diclofenac showed no potential to bioconcentrate, yet it affected fish metabolism by increasing cellular energy consumption in the muscle and consequently reducing fish net energy budget. The diverse response patterns evidence the need for future research focused on pharmaceuticals with different modes of action and their exposure effects on organismal physiological mechanisms and homeostatic status. Ultimately, the combination of sub-individual and individual responses is key for ecologically relevant assessments of pharmaceutical toxicity.

Keywords: Pharmaceuticals, Bioconcentration, Growth, Energy metabolism, Oxidative stress, Neurotoxicity

Introduction

Pharmaceutical compounds of human and veterinary use are often released into the aquatic environment, either directly or after incomplete removal by wastewater treatment plants, contributing to their continuous and persistent presence in many aquatic systems (Caldwell, 2016; Kümmerer, 2009). Hence, pharmaceuticals are commonly detected in surface, ground and drinking waters at concentrations in the ng/L and low µg/L range, yet maximum reported concentrations can reach hundreds of µg/L and up to mg/L (aus der Beek et al., 2016). Even if detected at low concentrations, these compounds may pose a risk to many species, as they are biologically active at very low concentrations and target specific pathways, most of them conserved throughout the tree of life, and in particular among vertebrates (Gunnarsson et al., 2008). Overall, pharmaceuticals have been found to affect various biological endpoints such as molecular and biochemical processes, including growth, metabolism, reproduction and behaviour (Duarte et al., 2019; Fabbri and Franzellitti, 2016; Sehonova et al., 2018). However, efforts have historically focused mainly on freshwater systems and acute exposure tests, with studies on chronical exposures and on estuarine and marine organisms still limited (Fent et al., 2006; Gaw et al., 2014; Reis-Santos et al., 2018).

With over 600 pharmaceuticals detected in the environment worldwide, therapeutic groups such as analgesics, antidepressants and anti-hypertensive drugs are prevalent (aus der

Beek et al., 2016). Within these classes, diclofenac (DCF), fluoxetine (FLX) and propranolol (PROP) are among the most used and prescribed drugs, and therefore some of the most frequently detected compounds in the aquatic environment, at concentrations ranging from ng/L to μg/L (aus der Beek et al., 2016; Bonnefille et al., 2018; Mezzelani et al., 2018). Diclofenac is a nonsteroidal anti-inflammatory drug (NSAID) commonly prescribed to treat pain, fever and inflammation, whereas fluoxetine belongs to the antidepressant class of selective serotonin reuptake inhibitors (SSRIs) used to treat depression and other psychiatric disorders, and propranolol is a beta-adrenergic receptor antagonist (β-blocker), used to treat hypertension and heart-related diseases. Although with varying environmental degradation rates and retention efficiencies in water treatment plants (Luo et al., 2014), their continuous release ultimately results in the permanent exposure of non-target species (Arnold et al., 2014; Monteiro and Boxall, 2010). In this context, chronic exposure assessments at environmental concentrations are paramount to address the potential risks posed by these compounds to aquatic species. In particular, examining the effects of pharmaceuticals with different modes of action (MOA), not yet fully described in fish, and at different levels of organization (i.e. sub-individual/biochemical and individual responses) will ultimately contribute to a more comprehensive and ecologically relevant assessment of pharmaceutical toxicity.

This study provides an integrative view on the risks and toxicity of three pharmaceutical compounds with different MOA, the antihypertensive PROP, the non-steroidal anti-inflammatory DCF, and the antidepressant FLX, in the meagre *Argyrosomus regius* (Asso, 1801), a top predator fish species of high economic value. The specific aim of this study was to assess the effects of long-term exposure to two distinct environmentally relevant concentrations, integrating different levels of biological organization. Thus, following exposure, alterations at the individual level were investigated, including fish growth, condition, and pharmaceutical bioconcentration. At the sub-individual level, various responses were assessed, namely: activity levels of antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD), involved in the detoxification of reactive oxygen species (ROS) thus reducing oxidative stress; the responses of biotransformation enzymes ethoxyresorufin O-deethylase (EROD) and glutathione-S-transferase (GST), responsible for the metabolism of xenobiotic compounds such as pharmaceuticals; the levels of oxidative stress effects such as lipid peroxidation (LPO), DNA damage (DNAd), and of neurotoxicity, acetylcholinesterase inhibition (AChE). Furthermore, energy-related parameters were assessed, including: the levels of each energy reserve (carbohydrates, proteins and lipids), and total energy available (EA); lactate dehydrogenase (LDH) and isocitrate dehydrogenase (IDH) enzyme activities, involved in anaerobic and aerobic metabolism pathways,

respectively, as well as the LDH/IDH ratio; the electron transport system (ETS) activity, a proxy for cellular energy consumption; and finally the cellular energy allocation (CEA), for the quantification of organismal energetic tradeoffs.

Materials and methods

Experimental design

Argyrosomus regius juveniles (7.31 ± 0.58 cm, 3.84 ± 0.83 g), obtained from a fish farm, were randomly distributed among 21 experimental 40 L tanks, with 8 individuals per tank, and acclimated to exposure conditions for 15 days. The long-term semi-static toxicity test was performed according to OECD guidelines (test no. 210) with a 16:8 h light:dark photoperiod and UV-treated natural seawater (average 24.8 PSU and 17.3 ºC). A control and two concentrations (low and high) were used for DCF, PROP and FLX exposures, with three replicate tanks per concentration. Fish were exposed for 30 days to nominal concentrations of 0.3 and 15 µg/L, for low and high concentrations, respectively. The exception was the high FLX treatment, which consisted of a separate 15 days' exposure to a 3 µg/L concentration with fish from the initial batch and with an independent control group (controls high FLX). This was due to early distress signs (swimming and feeding) evident within 48 h exposure in a preliminary test run with a 15 µg/L FLX concentration. Nonetheless, all concentrations used in this study cover the range of reported environmental concentrations for the different pharmaceutical classes (aus der Beek et al., 2016; Mezzelani et al., 2018).

Pharmaceutical stock solutions were prepared with milli Q-grade water and stored at –20ºC. Daily water renewals were performed (25%), and pharmaceutical concentrations appropriately restored to maintain nominal pharmaceutical concentrations in tanks. Water parameters, namely dissolved oxygen, temperature, salinity, pH, ammonia and nitrites, as well as any fish mortalities were recorded daily. Fish were fed daily with pellets developed for hatchery feeds (WinFast by Sparos), with portion adjustments throughout the experiment to maintain a 2% ratio with mean fish weight.

All experimental procedures were performed in accordance with animal testing guidelines (EU Directive 2010/63, Portuguese DL 113/2013), licensed by the animal welfare committee at the Faculty of Sciences of the Lisbon University, and by national authorities.

Growth and condition indices

Total fish length (Lt, in cm) and weight (Wt, in g) were recorded at the beginning and end of the experiment. Fulton's condition factor K was determined according to Ricker (1975): $K = Wt/Lt^3$, where Wt is total weight and Lt is total length. Specific growth rates in weight were determined per tank, in % per day, using the formula: $G = 100*(\ln Wt_f - \ln Wt_i)/(t_f - t_i)$, where Wt_f and Wt_i are fish total weights at final (t_f) and initial (t_i) days of exposure, respectively (Kroon et al., 2017).

Concentration of pharmaceuticals in water and fish tissues

Water samples were collected from each tank every week for pharmaceutical quantification. Sample extraction, purification, and concentration were adapted from Pereira et al. (2015) and Sousa et al. (2011). Samples (500 mL) were sequentially filtered through 3 membranes (1.2 µm, 0.45 µm and 0.2 µm), purified with OASIS HLB cartridges and subsequently washed with 5 mL of methanol:water (10:90) and eluted with 6 mL of methanol. The extract was dried under a gentle stream of N_2 at 40ºC. Prior to analysis, extracts were dissolved in 500 µL of methanol:water (3:97), filtered through a PVDF Mini-uniprep^{TM} filter (0.45 µm), injected and quantified through ultra-high performance liquid chromatography and time-of-flight mass spectrometry (UHPLC-TOF-MS). Results are presented as µg of pharmaceutical compound per liter of water.

Portions of fish dorsal muscle tissue (approximately 2 g) were sampled for pharmaceutical quantification, i.e. bioconcentration (expressed as µg/kg in fish tissue). Sample extraction, purification, and concentration were performed as an extension of the method from Freitas et al. (2014). Briefly, tissues were homogenized, and extraction was performed with 5 mL of acetonitrile and 1 mL of 0.1 M EDTA. Samples were centrifuged and the supernatant evaporated to near dryness (until 0.5 mL) under a gentle stream of N_2 at 40 ºC. After adding 500 µL of 0.1% formic acid to the residue, a filtration step through a PVDF mini-uniprep^{TM} filter (0.45 µm) was performed, followed by the injection into the UPHLC-TOF-MS for detection and quantification. Results are presented as µg of pharmaceutical per kg of wet weight. For a full description of methodology and instrumentation used for pharmaceutical quantification, including limits of quantification (LOQ) and recovery (%) in water and fish muscle samples, see Appendix 3, Table A3.1.

Biomarkers quantification

For biomarkers quantification different fish tissues were dissected, namely liver, brain, muscle, and heart. Tissue samples were homogenized in cold 100 mM monobasic potassium phosphate/dibasic potassium phosphate (K_2HPO_4/KH_2PO_4) buffer (pH 7.4) containing 0.15 M KCl (potassium chloride), 0.1 mM PMSF (phenylmethylsulfonyl fluoride), 1 mM DTT (dithiothreitol) and 1 mM EDTA (ethylenediaminetetraacetic acid) to avoid protein degradation. Liver homogenates were aliquoted for DNA damage (DNAd), lipid peroxidation (LPO) quantification, superoxide dismutase (SOD), catalase (CAT), ethoxyresorufin-*O*-deethylase (EROD) and glutathione *S*-transferase (GST) determination.

Muscle homogenates were used for determination of LPO, DNAd, electron transport system activity (ETS), lactate dehydrogenase (LDH) and isocitrate dehydrogenase (IDH) activities, as well as for determination of total carbohydrates (CBH), proteins (PT) and lipids (LP) content. Heart homogenates were used for ETS, LDH and IDH activity measurements. Brain homogenates were used for the measurement of acetylcholinesterase (AChE) activity.

All biomarker responses were determined using a Sinergy HT Microplate Reader (Bio-Tek Instruments, Vermont, USA), and each reading was done in triplicate using homogenization buffer as blank reaction. Superoxide dismutase (SOD) activity was measured according to Mccord and Fridovich (1969), and was expressed as U mg^{-1} of total protein concentration, where one unit is the amount of enzyme required to inhibit the reduction of cytochrome c by 50%. Catalase (CAT) activity was determined according to Aebi (1974), following substrate consumption, as a decrease in absorbance at 240 nm. CAT activity was then calculated as the difference in absorbance per unit of time ($\varepsilon = -0.04$ mM^{-1} cm^{-1}) and expressed as μmol per minute per mg of total protein concentration. Ethoxyresorufin-O-deethylase (EROD) activity was determined following Burke and Mayer (1974) method, with few adaptations by Fernandes et al. (2002). Activity was calculated as the amount of resorufin (ρmol) generated per mg of total protein per minute of reaction time. Glutathione S-transferase (GST) activity was determined following Habig et al. (1974), and activity was expressed as nmol CDNB conjugate formed per mg of total protein per minute of reaction. Lipid peroxidation (LPO) was determined according to Ohkawa et al. (1979) and was expressed as nmol of TBARS formed per mg of wet weight. DNA damage (DNAd) was determined following the DNA alkaline precipitation assay by Olive (1988). DNA concentration in the supernatant was determined following the addition of Hoechst dye and fluorescence values were compared to a DNA standard curve. DNAd was expressed as μg DNA per mg of wet weight. Acetylcholinesterase (AChE) was determined

according to Ellman et al. (1961), adapted to microplate (Guilhermino et al., 1996). The enzymatic activity was expressed in nmol of substrate hydrolyzed per minute per mg of total protein. LDH activity was assessed using the methods described by Vassault, (1983) and Diamantino et al., (2001) and results were expressed as nmol min^{-1} mg $protein^{-1}$ ($\varepsilon = 6.22 \times 10^3$ M^{-1} cm^{-1}). IDH activity was determined following Ellis and Goldberg, (1971) method, adapted by Lima et al., (2007), and results were expressed as nmol min^{-1} mg $protein^{-1}$ ($\varepsilon = 6.22 \times 10^3$ M^{-1} cm^{-1}). Aerobic and anaerobic pathways were also assessed through LDH/IDH ratio. Cellular energy allocation (CEA) was calculated as in Verslycke et al., (2004a, 2004b): CEA = Ea/Ec, where Ea (available energy) = carbohydrate + lipid + protein (mJ mg ww^{-1}), and Ec (energy consumption) = ETS activity (mJ h^{-1} mg ww^{-1}). Following De Coen and Janssen, (2003, 1997), total content of carbohydrates, lipids and proteins were measured and transformed into energetic equivalents using enthalpy combustion (39.5 kJ g^{-1} lipid, 24 kJ g^{-1} protein, 17.5 kJ g^{-1} glycogen, respectively). Results were expressed as mJ mg wet $weight^{-1}$. ETS activity in the mitochondria was determined according the method of De Coen and Janssen, (1997). Oxygen consumption was calculated using a stochiometrical relationship: 2 µmol of formazan formed = 1 µmol of oxygen consumed. The oxygen consumption rate was then converted into the energetic equivalent of 480 kJ mol O_2^{-1} for average carbohydrate, lipid, and protein consumption combinations (Gnaiger, 1983). Protein content was quantified following Bradford's method, adapted to microplate, and bovine serum albumin solution (1 mg mL^{-1}) was used as protein standard. For further protocol details see Appendix 3.

Data analyses

Differences in fish responses in DCF and PROP experiments were tested through permutational analyses of variance (PERMANOVA) followed by pair-wise tests (results presented as Pseudo-F and t_{pw}, respectively), whereas in FLX experiment, differences were tested with Mann-Whitney-Wilcoxon test (results presented as W). A multivariate nested design was initially considered, with treatment and tank treated as the fixed and nested (random) factors, respectively. Tank effects were absent for the majority of fish responses, except for 1 and 3 out of 25 responses analyzed for PROP and DCF treatments, respectively. Since no statistical differences were found when considering nested or one-factor design for these responses, we decided to use the less complex univariate design, with treatment as the fixed factor. Differences in specific growth rates (G) and pharmaceutical bioaccumulation were tested with Welch's t-test (results presented as t), considering its robustness when a reduced number of samples is

being tested (minimum N = 3). Spearman rank correlation (r) analysis was performed to test for correlations between fish responses. Analyses were performed in PRIMER 6 and R software (R Core Team, 2018), and a significance level of 0.05 was considered for all statistical tests used.

Results

Water quality parameters and pharmaceutical exposure concentrations

Water parameters were measured daily, and temperature (17.3 ± 0.2 ºC), salinity (24.8 ± 0.2), pH (8.1 ± 0.02) and dissolved oxygen (96.8 ± 0.1 %) were constant throughout the experiment, and ammonia and nitrite levels were maintained below 0.2 mg/L. Measured pharmaceutical concentrations were slightly lower than nominal concentrations, and evidenced low variation among measurements (Table 5.1). Average concentrations (µg/L) in the water were 0.13 and 9.25 for DCF; 0.15 and 2.52 for FLX and 0.27 and 14.74 for PROP, for low and high concentrations respectively (Table 5.1).

Table 5.1. Average (± standard deviation) concentrations of pharmaceuticals in water (µg/L) and in fish muscle (µg/kg) samples, for low and high treatments of fluoxetine (FLX), diclofenac (DCF) and propranolol (PROP).

	Water (µg/L)		Muscle (µg/kg)	
	Low	**High**	**Low**	**High**
FLX	0.15 ± 0.02	2.52 ± 0.27	66.3 ± 10.6	425.5 ± 215.8
DCF	0.13 ± 0.03	9.25 ± 1.18	< LOQ	< LOQ
PROP	0.27 ± 0.01	14.74 ± 2.65	1.39 ± 0.3	58.39 ± 22.8

Individual responses

Growth and condition indices

Mortality in all treatments and controls was lower than 10%, where two fish died in control and high FLX treatments, and one fish in low DCF treatment.

Fish length, weight and specific growth rates (G) were significantly reduced by FLX at the highest concentration ($W = 119.5$, $p < .05$; $W = 132$, $p < .01$; $t = 6.2$, $p < .01$, respectively; Fig. 5.1a-c), whereas Fulton's condition factor (K) showed the same pattern, yet without statistical significance ($W = 103$, $p > .05$; Fig. 5.1d). Contrarily, exposure to either low or high DCF

and PROP concentrations caused no significant morphometric changes in fish (Pseudo-F > 0.09, p > .05; Fig. 5.1), nor on growth rates (t > - 0.4, p > .05; Fig. 5.1c).

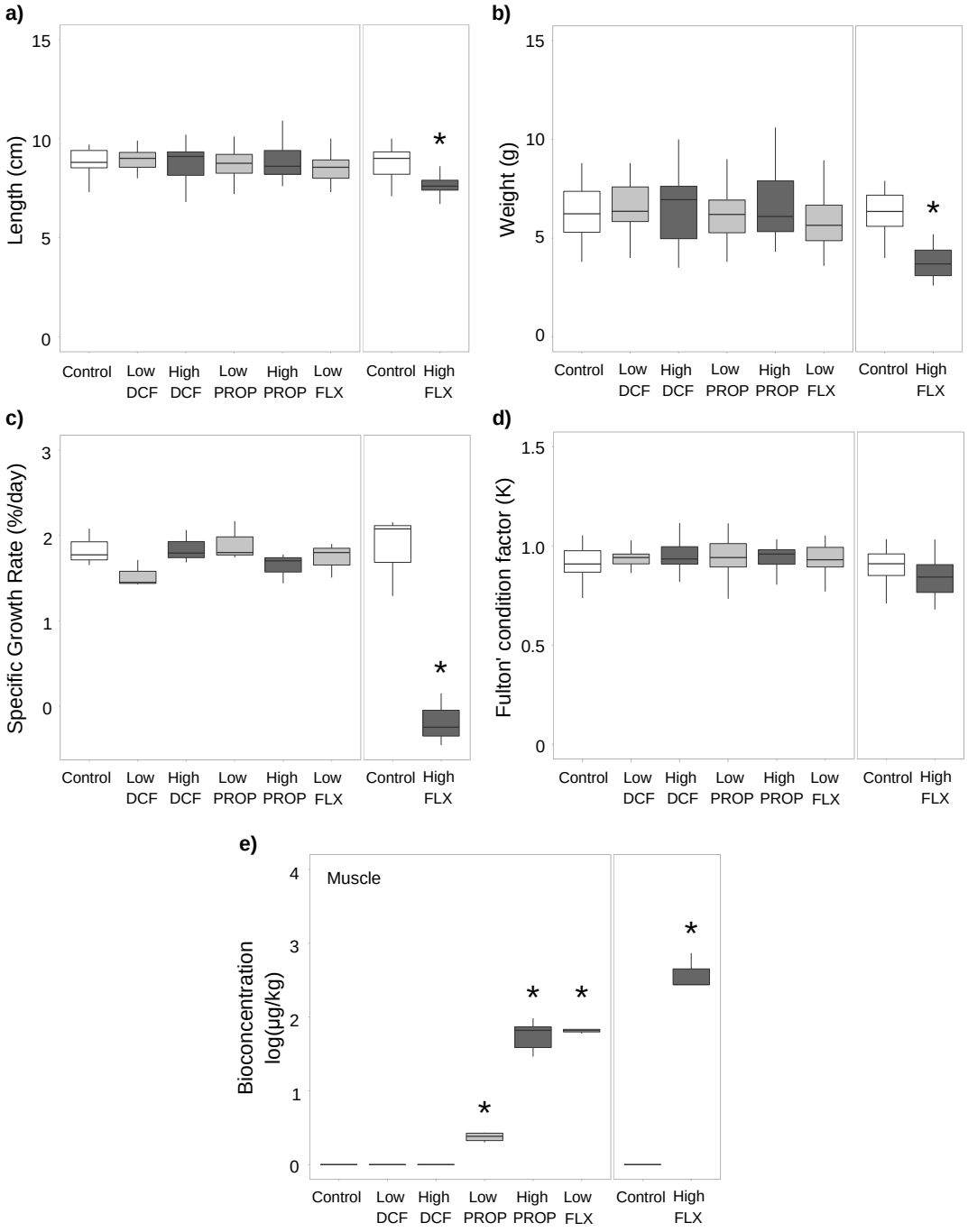

Figure 5.1. Individual responses of *Argyrosomus regius* juveniles after long-term exposure to low (light grey) and high (dark grey) concentrations of diclofenac (DCF), propranolol (PROP) and fluoxetine (FLX). Boxplots with median, 25th and 75th percentile (upper and lower whiskers represent 1.5 times the interquartile range (IQR) of maximum and minimum values, respectively) of responses measured: a) length (Lt), b) weight (Wt), c) specific growth rate (G), d) Fulton's condition factor (K) and e) bioconcentration in fish muscle. Asterisks indicate significant differences between treatments and respective controls. N=22-24 juveniles per treatment, except for FLX high (N=15) and respective control (N=10).

Pharmaceutical bioconcentration

Bioconcentration of pharmaceuticals in fish muscle tissues was observed for both low and high FLX concentrations ($t_{low\ FLX} = -77.2$, $p < .001$ and $t_{high\ FLX} = -18.1$, $p < .01$; Fig. 5.1e and Table 5.1) as well as for PROP ($t_{low\ PROP} = -15.3$, $p < .001$ and $t_{high\ PROP} = -23.5$, $p < .001$; Fig. 5.1e and Table 5.1), yet no bioconcentration was observed for DCF (Fig. 5.1e and Table 5.1).

Sub-individual responses

Fluoxetine

After long-term exposure to high FLX concentration, liver antioxidant enzyme SOD activity was significantly increased ($W = 13$, $p < .05$; Fig. 5.2a), whereas the same pattern was observed for CAT activity, but not statistically significant ($W = 14$, $p > .05$; Fig. 5.2b). Activity levels of biotransformation enzymes GST and EROD were significantly reduced after exposure to high FLX concentration ($W = 59$ and $W = 60$, $p < .05$, respectively; Fig. 5.2c and d). Low FLX concentration had no effects on antioxidant and biotransformation enzymes ($W > 206$, $p > .05$; Fig. 5.2).

Concerning damage, LPO levels were significantly reduced in the liver at low FLX concentration but increased at high concentration ($W = 357$, $p < .01$; Fig. 5.3a) and DNA damage was significantly increased at high FLX concentration ($W = 12$, $p < .05$; Fig. 5.3b). Contrarily, no changes in LPO and DNA damage were observed in muscle at low concentration ($W > 251$, respectively, $p > .05$; Fig. 5.3c and d) and no neurotoxic effects, namely changes in acetylcholinesterase activity, were observed in fish brain at both FLX concentrations ($W > 51$, $p > .05$; Fig. 5.3e).

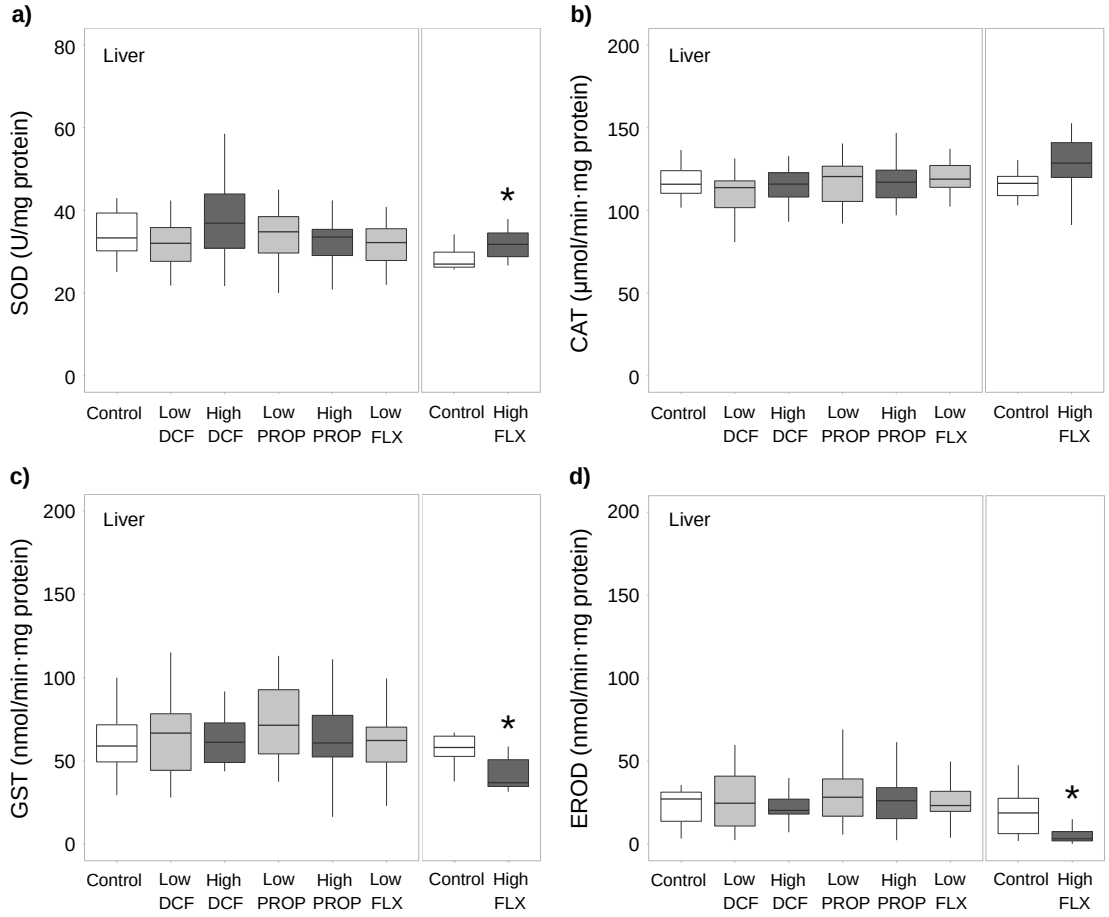

Figure 5.2. Biomarker responses of *Argyrosomus regius* juveniles after long-term exposure to low (light grey) and high (dark grey) concentrations of diclofenac (DCF), propranolol (PROP) and fluoxetine (FLX). Boxplots with median, 25th and 75th percentile (upper and lower whiskers represent 1.5 times the interquartile range (IQR) of maximum and minimum values, respectively) of enzymes' activities measured in the liver: a) superoxide dismutase (SOD), b) catalase (CAT), c) glutathione S-transferase (GST) and d) ethoxyresorufin-O-deethylase (EROD). Asterisks indicate significant differences between treatments and respective controls. N=22-24 juveniles per treatment, except for FLX high (N=15) and respective control (N=10).

FLX did not affect fish heart nor muscle energetic metabolism, i.e., aerobic and anaerobic pathways, assessed through LDH/IDH ratio ($W > 157$, $p > .05$; Appendix 3, Fig. A3.1; Fig. 5.4a). Moreover, the amount of energy reserves available (EA, i.e. total sugar, protein and lipids) and the electron transport system (ETS) activity, a proxy for energy consumption, also remained unchanged in muscle at low FLX concentration ($W > 137$, $p > .05$; Fig. 5.4b and Appendix 3, Fig. A3.2) which, consequently, revealed no significant changes in cellular energy allocation (CEA) ($W = 152$, $p > .05$; Fig. 5.4c). Few significant correlations could be observed among fish responses to FLX (Appendix 3, Table A3.4). Muscle and heart ETS activity were negatively correlated with both fish length and weight (Muscle: $r = -0.40$, $p < .01$ and $r = - 0.34$, $p < .05$, respectively. Heart: $r = -0.32$ and $r = - 0.32$, $p < .05$, respectively).

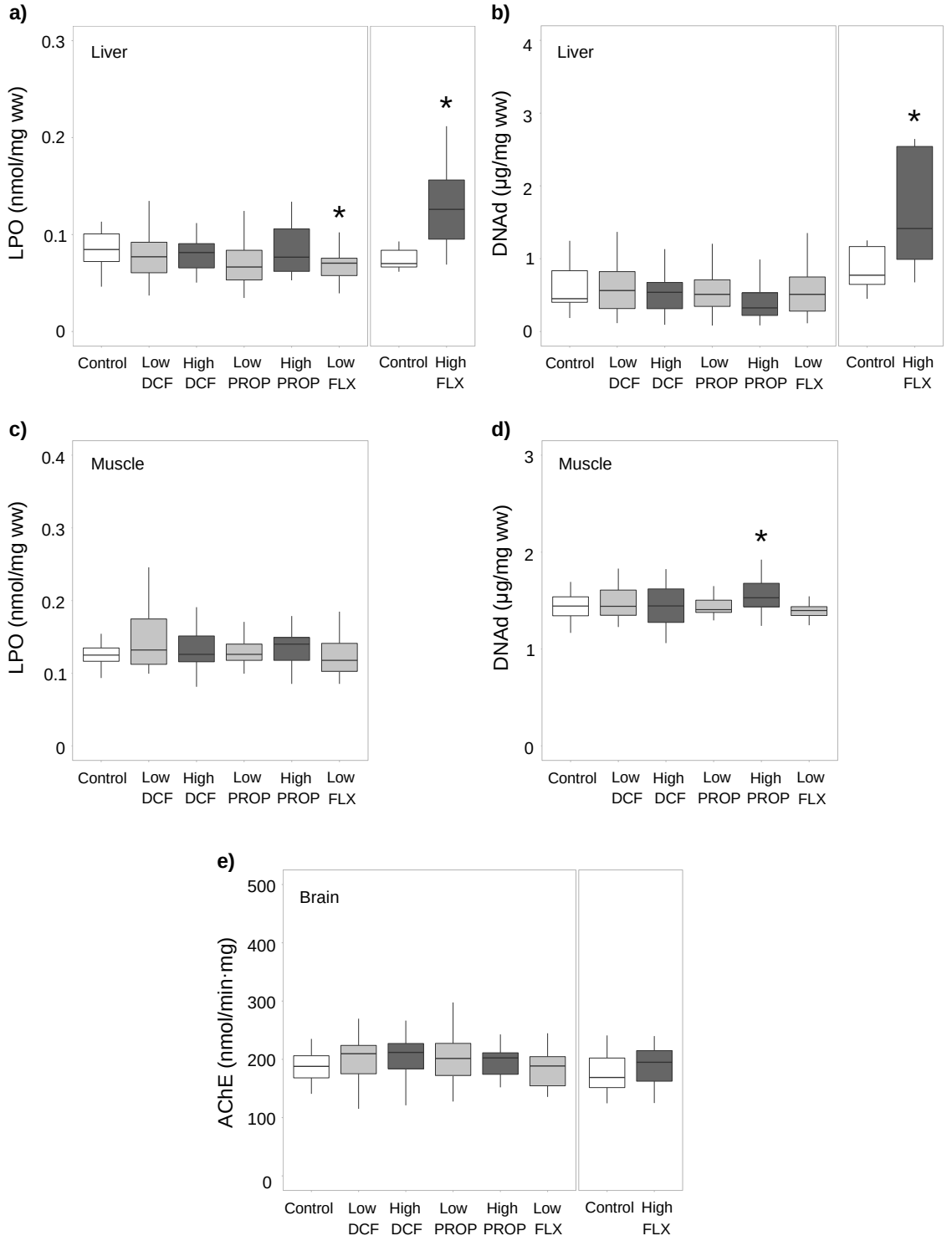

Figure 5.3. Oxidative and neurotoxic effects of *Argyrosomus regius* juveniles after long-term exposure to low (light grey) and high (dark grey) concentrations of diclofenac (DCF), propranolol (PROP) and fluoxetine (FLX). Boxplots with median, 25th and 75th percentile (upper and lower whiskers represent 1.5 times the interquartile range (IQR) of maximum and minimum values, respectively) of effects measured in the liver, muscle and brain: a) liver lipid peroxidation (LPO), b) liver DNA damage (DNAd), c) muscle lipid peroxidation (LPO), d) muscle DNA damage (DNAd) and e) brain acetylcholinesterase activity (AChE). Asterisks indicate significant differences between treatments and respective controls. N=22-24 juveniles per treatment, except for FLX high (N=15) and respective control (N=10).

Diclofenac

Long-term exposure to DCF caused no significant effects in liver antioxidant enzymes SOD and CAT and in biotransformation enzymes GST and EROD activities (Pseudo-F > 0.002, p > .05; Fig. 5.2a – d), as well as no damage to liver and muscle lipids and DNA, nor to brain AChE activity (Pseudo-F > 0.2, p > .05; Fig. 5.3a – e). However, DCF significantly increased ETS activity in fish muscle at both low and high concentrations (Pseudo-F = 3.9, p < .05; Fig. 5.4b), but not in the heart (Pseudo-F = 0.38, p > .05; Appendix 3, Fig. A3.1). Since muscle energy reserves were unaffected (Pseudo-F > 0.19, p > .05; Appendix 3, Fig. A3.2), a significant reduction in fish net energy budget (CEA) was observed following the increase in ETS (Pseudo-F = 5.1, p < .01 and Pseudo-F = 3.88, p < .05, respectively; Fig 5.4a and b), yet with no significant changes to LDH/IDH ratio (Pseudo-F = 3.2, p > .05; Fig. 5.4c). Few significant correlations among biomarker responses were observed (Appendix 3, Table A3.5).

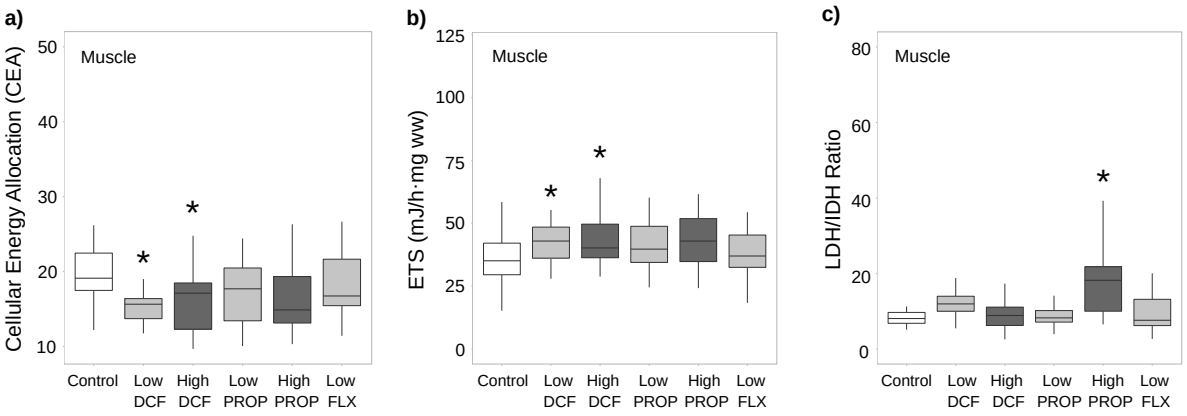

Figure 5.4. Energy related responses of *Argyrosomus regius* juveniles after long-term exposure to low (light grey) and high (dark grey) concentrations of diclofenac (DCF), propranolol (PROP) and fluoxetine (FLX). Boxplots with median, 25th and 75th percentile (upper and lower whiskers represent 1.5 times the interquartile range (IQR) of maximum and minimum values, respectively) of responses measured in fish muscle: a) cellular energy allocation (CEA), b) electron transport system activity (ETS) and c) LDH/IDH ratio. Asterisks indicate significant differences between treatments and respective controls. N=22-24 juveniles per treatment, except for FLX high (N=15) and respective control (N=10).

Propranolol

Exposure to PROP caused no changes in liver antioxidant CAT and SOD enzymes and biotransformation GST and EROD enzymes responses (Pseudo-F > 0.004, p > .05: Fig. 5.2a – d). Also, no effects on lipids and DNA damage were observed in the liver (Pseudo-F > 1.82, p > .05; Fig. 5.3a and b), whereas in muscle, DNA damage was significantly increased at high PROP concentration (t_{pw} = 2.2, p < .05; Fig. 5.3a – d). No neurotoxicity was found at both concentrations (Pseudo-F = 0.7, p > .05; Fig. 5.3e).

Muscle metabolic ratio LDH/IDH was significantly increased after exposure to high PROP concentration (t_{pw} = 4.3, p < .001; Fig. 5.4c) due to a decrease in aerobic metabolism, i.e. IDH activity (Pseudo-F = 14.68, p < .01; Appendix 3, Fig. A3.2). However, no significant changes in energy reserves or ETS activity followed PROP exposure (Pseudo-F > 1.04, p > .05; Appendix 3, Fig. A3.2; and Fig. 5.4b), hence CEA also remained unchanged (Pseudo-F = 2.1, p > .05; Fig. 5.4a). Correlations among *A. regius* responses were observed (Appendix 3, Table A3.6). In the heart, LDH was negatively correlated with fish length and weight (r = – 0.30, p < .05 and r = – 0.37, p < .01, respectively).

Discussion

Exposure to pharmaceuticals from different therapeutic groups at environmentally relevant concentrations had distinct effects in juvenile meagre *Argyrosomus regius*. Fluoxetine (FLX) was the most toxic pharmaceutical of the three, affecting fish growth, increasing antioxidant response, inhibiting liver biotransformation enzymes and triggering lipid peroxidation and DNA damage in the liver. On the other hand, Diclofenac (DCF) affected fish metabolism, by increasing cellular energy consumption in the muscle and reducing fish net energy budget. Effects of Propranolol (PROP) exposure were observed only at high concentration in muscle, where DNA damage increased, and a higher energy demand caused a shift to anaerobic metabolism.

Individual responses

Growth and condition

Exposure to FLX (3 µg/L) resulted in decreased length, weight and growth rate in juvenile *A. regius*. Few earlier studies have also showed decreased fish growth after long-term waterborne exposure to FLX in the µg/L range (0.03 to 200 µg/L) (Mennigen et al., 2010; Pelli and Connaughton, 2015), yet no effects on growth, condition or weight have also been reported (Chen et al., 2018; Foran et al., 2004). These different responses are likely associated with interspecies differences in metabolic efficiency (Smith et al., 2010), albeit this study is the first to consider a brackish-marine species. Nonetheless, FLX effect on fish growth and condition may be linked to serotonin-mediated appetite suppression as well as to altered behaviours (McDonald, 2017).

Detrimental effects of PROP on fish growth have been reported at much higher concentrations (above 500 µg/L) (Giltrow et al., 2009; Huggett et al., 2002; Owen et al., 2009).

Accordingly, no significant effects of PROP in fish length, weight, condition or growth rates, were found in the present work, even though a slight decrease in growth rate could be perceived at high concentration, it was not statistically significant. Accordingly, these results suggest that PROP may not likely affect fish growth or condition at environmentally relevant concentrations.

Similarly, long-term exposure to high DCF concentrations was shown to decrease fish weight and growth rates (Memmert et al., 2013; Praskova et al., 2014), whereas lower concentrations caused no significant effects on fish length, weight, growth rates or condition (Lee et al., 2011). As discussed for PROP, our results suggest that DCF exposure do not affect meagre morphometrics at present environmentally relevant concentrations.

Pharmaceutical bioconcentration

Bioconcentration in fish muscle differed between the three pharmaceuticals tested, likely due to differences in biotransformation capacity. The metabolism of these three pharmaceuticals has been tested in fish, both *in vitro* (e.g. Baron et al., 2017; Connors et al., 2013; Smith et al., 2012) and *in vivo* (e.g. Ding et al., 2015; Lahti et al., 2011; Margiotta-Casaluci et al., 2014). Yet, differences in metabolic rates were demonstrated *in vitro* by Connors et al. (2013), where extensive metabolism of PROP and DCF by fish hepatocytes was observed, whilst FLX was not metabolized, therefore supporting greater potential for bioaccumulation. Likewise, *in vivo*, accumulation of FLX but not DCF was observed in rainbow trout (Zhang et al., 2010), and higher PROP bioconcentration, when compared to DCF, was described in zebrafish embryos (Bittner et al., 2019). Similarly, in this study, FLX was noticeably bioconcentrated in fish muscle, in comparison to PROP and DCF. FLX uptake in fish occurs within few hours of exposure (Paterson and Metcalfe, 2008), and it bioconcentrates in different tissues (Nakamura et al., 2008; Pan et al., 2018; Schultz et al., 2011). Moreover, its concentration in fish tissues increases with exposure time (Ding et al., 2016), probably due to the low biotransformation rates reported for FLX.

Likely as a result of efficient metabolism and depuration, DCF has low potential for bioconcentration in juvenile fish (Memmert et al., 2013; Schwarz et al., 2017), as observed in low or untraceable muscle DCF concentrations of fish exposed under controlled conditions to similar ranges of concentrations (µg/L) (e.g. Daniele et al., 2016; Memmert et al., 2013). On the other hand, there is limited information regarding PROP bioconcentration in fish. Uptake of PROP into fish blood plasma has been reported (e.g. Bartram et al., 2011; Giltrow et al., 2009; Owen et al., 2009), however, with low ensuing PROP bioconcentration in muscle tissues

(Ding et al., 2015), probably due to rapid and efficient PROP metabolism, as observed *in vitro* by several authors (Baron et al., 2017; Connors et al., 2013; Gomez et al., 2010). In fact, decreasing PROP concentrations in fish tissues with exposure time (Ding et al., 2016, 2015) further corroborates efficient PROP metabolism in fish.

Sub-individual responses

Antioxidant and biotransformation enzymes

FLX toxicity in fish hepatocytes has been linked to increased reactive oxygen species (ROS) production but also to its inhibitory effect on biotransformation enzymes of the cytochrome P450 family, including EROD (Fernández et al., 2013; Laville et al., 2004). Likewise, *in vivo* inhibition of biotransformation enzymes GST and EROD by FLX has previously been reported (e.g. Chen et al., 2018; Ding et al., 2016), as well as increased CAT and SOD activities at low μg/L concentrations (Pan et al., 2018). Accordingly, in this study exposure to high FLX treatment also triggered an increased antioxidant response and inhibited both biotransformation enzymes activities, revealing enhanced oxidative stress and FLX toxicity at environmentally relevant concentrations. Likewise, SNRI venlafaxine, sharing the same mode of action of FLX, also increased liver CAT activity and inhibited GSTs in *A. regius* juveniles after 28 days of waterborne exposure at 20 μg/L (Maulvault et al., 2018b).

Contrarily to FLX, Laville et al., (2004) found that DCF did not increase ROS production in fish hepatocytes, whilst increased DCF exposure ensued fish antioxidant responses only at higher DCF concentrations (high μg/L to mg/L range) (e.g. Islas-Flores et al., 2013; McRae et al., 2019; Pandey et al., 2017). Moreover, either no changes or inhibition of EROD enzyme activity were reported *in vitro* (Laville et al., 2004; Thibaut et al., 2006) and *in vivo* (Guiloski et al., 2017; Prokkola et al., 2015), with GST induced after long-term exposure to similar concentrations but inhibited at higher μg/L (Guiloski et al., 2017; Stancova et al., 2017). In this study, no differences in liver antioxidant and biotransformation enzymes were found after exposure to DCF, suggesting low potential to cause oxidative stress in *A. regius* at environmental concentrations tested. Similar results were also observed by Maulvault et al., (2018) in top predator *Dicentrarchus labrax* juveniles after dietary exposure to DCF.

PROP also failed to increase ROS production in fish hepatocytes (Laville et al., 2004). Yet, only a few studies have measured antioxidant and biotransformation enzymes responses *in vivo*, without significant alterations after waterborne exposure (Bartram et al., 2011; Pereira

et al., 2018). Likewise, we observed no changes on antioxidant and biotransformation enzymes activities, suggesting low PROP toxicity at tested concentrations.

Oxidative damage and neurotoxic effects

In line with the enzymatic responses described above, FLX exposure (3 μg/L) resulted in both LPO and DNA damage in the liver of juvenile meagre. Similarly, exposure to FLX in the μg/L range have been shown to increase LPO levels in *Carassius auratus* and *Pseudorasbora parva* after 7 and 42 days, respectively (Chen et al., 2018; Ding et al., 2016), whilst no significant effects were observed in *Pomatoschistus microps* after only 4 days (Duarte et al., 2019). Increased lipid peroxidation was also observed in *A. regius* juveniles after 28 days of waterborne exposure to venlafaxine (20 μg/L), another serotonin reuptake inhibitor (Maulvault et al., 2018b). To the best of our knowledge, genotoxic effects of FLX in fish were firstly assessed in our previous study (Duarte et al., 2019), with no changes in DNA damage reported for *Pomatoschistus microps* after 4 days of exposure to μg/L concentrations and 1 h exposures to mg/L range concentrations. Ultimately, LPO and DNA damage likely occur after longer exposure periods, supporting the potential for FLX to promote oxidative and genotoxic effects in the long term, even at low concentrations.

FLX can modulate fish cholinesterase activity and ultimately alter fish behaviour, with AChE activity pointed out as a valuable biomarker to study FLX neurotoxicity even if the mechanisms of this interaction are still unclear (e.g. Duarte et al., 2019; Farias et al., 2019). No significant differences in AChE activity were observed in *A. regius* juveniles, yet a slight increase at high FLX treatment could be perceived, which is in agreement with previous studies (Chen et al., 2018; Pan et al., 2018). However, inhibition or no effects in acute exposures have also been reported (Duarte et al., 2019; Farias et al., 2019). Differences in exposure duration, concentrations tested, as well as species and life-stages considered are likely the cause for such varying responses, and evidence the need for further investigation on the impact of FLX on fish neurological pathways.

Environmental DCF concentrations did not generate obvious oxidative stress in *A. regius* juveniles. Similarly, no DNA damage was previously observed in *Rhamdia quelen* liver after acute and chronic exposures to a comparable range of concentrations (Ghelfi et al., 2016; Guiloski et al., 2017), though increased DNA damage in *O. niloticus* juveniles was observed after exposure to much higher concentrations (mg/L) of DCF for 15 days (Pandey et al., 2017). Few studies have measured the long-term effects of waterborne DCF exposure to lipid peroxidation in fish yet these report inexistent (Schwarz et al., 2017; Stancova et al., 2017) or

decreased (Guiloski et al., 2017) levels of LPO. After long-term dietary exposure to DCF, Maulvault et al., (2018a) also reported inexistent lipid peroxidation in *Dicentrarchus labrax* juveniles.

Concerning PROP, two studies have measured lipid peroxidation *in vivo* in fish after dietary exposure, where no differences in LPO levels were found (Ding et al., 2016, 2015). Similarly, in this study, waterborne exposure to PROP did not trigger lipid peroxidation in *A. regius*, but increased DNA damage in fish muscle, which, to the best of our knowledge, is the first record on genotoxic damage of this pharmaceutical in fish. Furthermore, only Pereira et al. (2018) have assessed the effects of PROP on cholinesterase activities in freshwater fish *Phalloceros harpagos* and, in agreement with our results, found no significant differences after acute and chronic exposures to a wide range of μg/L concentrations.

Energy metabolism

Disruption of fish energy metabolism by low μg/L FLX concentrations has been previously acknowledged (Mennigen et al., 2010; Mishra et al., 2017). However, no other studies have to date specifically addressed the effects of FLX on LDH and IDH enzymes activities, nor ETS activity in fish. Contrarily to previous findings, fish energy metabolism in *A. regius* juveniles' muscle and heart was not affected by FLX. Nonetheless, potential links between decreased fish growth and muscle energy metabolism cannot be discarded, especially considering the negative correlations observed between heart and muscle ETS activity and fish length and weight. Although FLX had no impact on energy metabolism at low concentration, the cost of FLX exposure in fish metabolic performance merits further investigation.

DCF clearly affected fish energy metabolism through an increase in muscle ETS activity at both concentrations and via a significant reduction of CEA. As a measure of cellular oxygen consumption and metabolism (King and Packard, 1975), increased ETS levels suggests that even at the lowest concentration tested, DCF significantly increased energy expenditure in *A. regius* juveniles. Interestingly, this increase in energy demand and consequent reduction of net energy budget (CEA) was not sufficient to bring changes in energy reserves (proteins, lipids and carbohydrates). Similarly, no changes in liver LDH activity were reported in *Gasterosteus aculeatus* exposed to 1 μg/L for 14 days (Lubiana et al., 2016), and increased LDH activity in serum and gills of *Clarias gariepinus* juveniles was found only after exposure to concentrations in the mg/L range (Ajima et al., 2015).

On the other hand, PROP exposure increased muscle LDH/IDH ratio, due to a decrease in muscle IDH activity. IDH is involved in cellular energy production via the aerobic pathway, but it is also a key enzyme to maintain cellular defence mechanisms (Jo et al., 2001). Since mitochondrial energy production, i.e. ETS activity, was not affected by PROP, this reduction in IDH activity might indicate a decreased capacity to counteract PROP oxidative stress in the muscle, which is further revealed by the increased DNA damage levels at the high concentration. Nonetheless, the slight decrease in growth rate observed at high PROP concentration, might be linked to this shift to anaerobic metabolism, along with the slight increase in ETS, i.e. energy consumption, hence reducing the amount of energy available for somatic growth. Indeed, this interpretation is also supported by the negative correlations observed between heart LDH activity and both fish length and weight. Accordingly, previous studies have reported inhibition of metabolic processes such as glycogen production and glucose release in rainbow trout as a result of PROP binding to hepatic b-adrenoceptors (Fabbri et al., 1998; Gesto et al., 2014), yet further investigation is needed to clarify the impact of PROP in fish metabolism.

Conclusions

Overall, pharmaceuticals from different therapeutic classes caused distinct responses in *A. regius* juveniles. FLX was the most toxic pharmaceutical of the three, impairing fish growth and liver biotransformation mechanisms. Additionally, oxidative stress observed in FLX treatment appears to be triggered by the combination of inhibited biotransformation mechanisms and prominent bioconcentration in fish muscle which is further emphasized by the increase in LPO and DNA damage in the liver. No changes in energy metabolism were found at low FLX concentration, yet effects at high concentration evidenced fish growth impairment and should be considered in future studies, given that FLX concentrations in the low μg/L range are environmentally relevant.

PROP and DCF exposure caused no effects on fish growth or condition, yet PROP bioconcentrated in muscle tissues, whilst DCF was not detected. Moreover, DCF did not enhance oxidative stress in fish liver, yet increased fish energy consumption in muscle, although not sufficient to cause changes in metabolic strategy or energy reserves. Interestingly, PROP caused a significant decrease in IDH activity, may be related to its role in cellular antioxidant defence, suggesting increased oxidative stress in muscle, as revealed by DNA damage increase.

Overall, each pharmaceutical generated different individual and biochemical responses. Specifically, FLX higher toxicity was evident in changes at the individual level as well as in biochemical changes in fish liver, whereas PROP and DCF effects were observed on

biochemical changes in fish muscle. Although the modes of action of these pharmaceuticals are not fully described in fish, these may be the cause for differences in responses observed in this study. An Omics approach could give further insight into the mechanisms underlying pharmaceuticals' toxicity in fish, including those related to biotransformation, oxidative stress, as well as detrimental effects on fish growth and energy metabolism. Ultimately, future research addressing the impacts of pharmaceuticals in different fish species to evaluate how physiology, behaviour and ecology underpin inter specific differences in effects, is key to improve our understanding of the environmental risk posed by pharmaceuticals.

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CHAPTER 6

General discussion and future perspectives

General discussion and future perspectives

Pharmaceutical contamination is a recent yet well-recognized environmental issue particularly in aquatic environments. Their pervasiveness, continuous-release, and potential to generate adverse effects even at low concentrations, may pose a severe threat to aquatic organisms, with future scenarios considering increased environmental concentrations due to predicted increased population and access to therapeutic compounds. In this context, this thesis provides new insights into the occurrence and bioaccumulation patterns of neuroactive pharmaceuticals in estuaries, as well as on the effects of exposure on non-target fish species through an integrated analysis of multi-biomarkers at the sub-individual and individual levels. The work developed here significantly advances current knowledge on environmental fate and toxicity towards a more comprehensive and effective risk assessment of the impacts of these compounds in estuarine environments.

A decade ago, the first studies raising attention to the problem of pharmaceutical contamination in the marine environment were published, combining the first scientific findings concerning deleterious effects in non-target species (Fabbri and Franzellitti, 2016; Gaw et al., 2014). Ever since the impacts of pharmaceuticals from different therapeutic classes have been investigated and increasing evidence of threat to non-target species revealed, a few neuroactive compounds (e.g. antidepressants, anxiolytics) have been at the forefront of reviews, albeit mostly in a descriptive way (e.g. Calisto and Esteves, 2009; Cunha et al., 2019, 2017; Sehonova et al., 2018). Hence, the work developed in Chapter 2, reviewing 451 studies encompassing 87 neuroactive pharmaceuticals, underpins the increasing examination of the potential for bioconcentration and toxicity of neuroactive pharmaceuticals in fish, and importantly, the identification of major trends and knowledge gaps that should be addressed in future studies. Notably, Chapter 2 revealed a skewed set of data: the bulk of toxicological studies were mostly limited to the investigation of antidepressants (largely fluoxetine), two anaesthetics (tricaine mesylate and benzocaine) used in aquaculture, and carbamazepine, one of the most ubiquitous pharmaceutical in the environment. While the rationale of focusing research on these compounds is reasonable and reflects some critical and first findings on this field, it evidences the need to expand research to a broader range of neuroactive pharmaceuticals, many of which their toxicity is unknown. The gap between freshwater and brackish or marine studies also remains considerably large, although some years have passed since the first calls for action (Fabbri and Franzellitti, 2016; Gaw et al., 2014) and suggestions of higher risk for coastal and marine species (e.g., Minguez et al., 2016). Coastal species could be particularly vulnerable to

pharmaceutical exposure, given the urban development in the vicinity of estuarine and coastal areas. A variety of fish species rely on estuaries to complete their life cycle, using these ecosystems as passage (e.g. diadromous), over specific life-stages (e.g. marine migrants) or throughout their lifespan (estuarine species), which implies that they are well-adapted to these naturally dynamic environments, on top of anthropogenic stressors (Elliott and Dewailly, 1995; Elliott and Quintino, 2007; Franco et al., 2008). Still the impacts of pharmaceuticals are being vastly driven by information on freshwater species, and largely targeting up to four freshwater species (Chapter 2), limiting the knowledge on the impacts of pharmaceutical contamination in estuarine and marine species.

In toxicology, the analysis of early-warning signs, referred to as biomarkers, allows for detecting premature alterations at different levels of biological organization, that can signal exposure, effects or susceptibility to any contaminant, and it is well recognized by the scientific community as a preeminent tool for environmental quality assessment (e.g. van der Oost et al., 2003). Accordingly, the toxicity of neuroactive pharmaceuticals was investigated through molecular changes and through alterations to fish behaviour, physiology, growth and reproduction (Chapter 2), which are of high ecological relevance. The sensitivity of these endpoints was evident, where the majority of MRC (minimum response concentrations, i.e. the lowest concentration at which a significant effect was observed) corresponded mostly to changes in behaviour, but also growth and condition or reproduction, undoubtedly more sensitive and therefore more environmentally relevant than other endpoints such as mortality (e.g. Melvin and Wilson, 2013). These outcomes highlight the importance of including sensitive individual-level endpoints in future toxicity assessments of neuroactive compounds, particularly behavioural endpoints, which are central considering the mode of action of neuroactive pharmaceuticals, with increased potential to interfere with key neuronal processes and consequently behaviour.

The toxicity of contaminants is directly linked to their uptake and accumulation in biota tissues, and the effects of such exposure are expected to occur when critical internal concentrations are reached (Sijm and Hermens, 2005). In non-target biota, effects of exposure to pharmaceuticals are expected if human drug targets are conserved, according to the read-across hypothesis (Huggett et al., 2003), which is mostly the case with fish species (Gunnarsson et al., 2019). Therefore, if the accumulated concentration surpasses human therapeutic concentrations, it will define a pharmacological or toxicological response which is, theoretically, expected to increase with increasing internal concentrations. Indeed, the results from Chapter 2 confirmed that neuroactive pharmaceuticals with higher bioconcentration potential are the most lethal, i.e. pharmaceuticals that bioconcentrate more will reach a critical internal concentration

(the lethal body burden) at lower concentrations, after which the death of the organism is certain (Sijm and Hermens, 2005). Yet, the same relation was not clear for the remaining toxicity endpoints, namely growth and condition, behaviour, and reproduction. The reason behind these non-significant correlations is most likely related to the fact that few studies determining toxicity endpoints have concurrently assessed internalized pharmaceutical concentrations. This inevitably results in comparing BCFs and biological responses arising from different studies (constrained to their specificities, such as life-stages, species or tissues), therefore hampering the link between bioconcentration and toxicity (McCarty et al., 2011; Rand-Weaver et al., 2013). In the few cases where this approach was considered, however, it is evident that significant effects are associated with increased internal concentrations (e.g. Pan et al., 2018; Xie et al., 2015). This reflects the absolute need to integrate both toxicity responses and internalized concentration measurements, excluding a significant source of inter-studies variability, and therefore contributing for a straightforward link between exposure and risk/toxicity assessments.

The potential for bioconcentration is usually determined through compounds' lipophilicity ($logK_{ow}$), which defines their propensity to partition into lipidic fractions, in this case organisms' tissues/membranes. Higher lipophilicity is thus usually akin to higher accumulation up to $logK_{ow}$ 6, and this relation has been confirmed for other chemical compounds (e.g. Arnot and Gobas, 2006; Bintein et al., 1993). Therefore, lipophilicity has been used in European guidelines as a threshold for evaluating bioaccumulation potential and for environmental risk assessment (ERA) of existing and newly designed chemicals and pharmaceuticals (e.g. $logK_{ow} > 3$ by European Chemicals Agency, ECHA; $logK_{ow} > 4.5$ by European Medicines Agency, EMA). Most neuroactive pharmaceuticals fall into the category of lipophilic compounds with low molecular weight, a suitable combination for increased permeability needed to cross the blood-brain barrier (Pardridge, 2007). However, the suitability of $logK_{ow}$ as a predictor of bioconcentration for neuroactive pharmaceuticals was still unknown. In Chapter 2, it was evident that predicting bioaccumulation potential exclusively through lipophilicity may not be the best approach for neuroactive pharmaceuticals, revealing how compounds with high bioaccumulation factors can be excluded by application of current guidelines. Likewise, this relation is also unclear when data is narrowed down to specific therapeutic classes (e.g. antidepressants or psycholeptics) or to specific tissues (e.g. brain, plasma or others, except for whole-body analysis). Therefore, current threshold regulations should be adjusted to include the risk assessment of compounds with lipophilicity lower than 3 and, ideally, bioaccumulation and toxicity studies should be conducted for all new compounds, independently of their $logK_{ow}$, with decisions based on empirical data. Despite risk assessment results not being accounted for in the final

decision on marketing authorization, it can be useful for providing detailed analysis concerning their impacts to the environment, particularly on the fate, toxicity and biodegradability of these compounds, and ultimately mitigation measures. Likewise, the revision of ERA for pharmaceutical compounds that entered the market before legislation applied (in 2006), for which risk assessment is missing, is critical, and has been proposed as a requirement for improving environmental risk assessment of pharmaceuticals (Ågerstrand et al., 2015).

The high variability of bioconcentration factors (BCF) was also evident, which, besides the inherent particularities of neuroactive pharmaceuticals, can be due to multiple factors such as between-studies variability, differences in experimental parameters (exposure time, temperature, salinity and pH) but also inter-species, life-stages and tissues dissimilarities (Arnot and Gobas, 2006; Sijm and Hermens, 2005). In the case of neuroactive pharmaceuticals, only a few studies have addressed the influence of abiotic parameters (e.g. McCallum et al., 2019; Nakamura et al., 2008; Xie et al., 2015) and studies directly comparing species-specific responses or the influence of life-stages are notably insufficient, but appear to support BCF variations accordingly (e.g. Heynen et al., 2016; Pan et al., 2018). A highly important factor in exposure is the time to reach steady-state (i.e. the equilibrium between external and internal concentrations), which varies among studies, with several studies reporting higher BCFs over time, at lower concentrations and in the brain (Pan et al., 2018; Xie et al., 2015; Ziarrusta et al., 2017), which is critical for addressing the impacts of neuroactive compounds. Notably, tissue selection plays a critical role in BCF determination, with considerable higher BCFs reported in brain and liver tissues, in comparison to muscle tissues or whole-body measurements, which may contribute to the under estimation of internalized concentrations. The use of plasma measurements has also been proposed as a valuable tool, considering the direct link with human therapeutic concentrations (Huggett et al., 2003), yet no clear patterns were evidenced and its use in research is still limited to a few compounds. The complexity of toxicokinetic and toxicodynamic processes is well known (McCarty et al., 2011), and evidently not an exception for neuroactive pharmaceuticals, even under controlled conditions. Some works report intra and interspecies influences in fish (e.g. Villeneuve et al., 2010; Vossen et al., 2020), yet more research is needed to investigate the influence of such factors. At the same time, an effort towards increased standardization and calibration of experimental settings is crucial towards improving information for regulatory frameworks.

Finally, this chapter highlighted nine compounds of priority concern, that are either exceeding or close to reach toxic concentrations for fish in their natural environments. By comparing the lowest concentrations known to affect fish (MRC) with currently detected

concentrations in freshwaters or brackish and marine waters, it is evident that at least nine neuroactive compounds (namely fluoxetine, citalopram, sertraline, amitriptyline, venlafaxine, clozapine, carbamazepine, metamfetamine and oxazepam) should be prioritized in future studies and included in risk management strategies and regulations. Research and prioritization efforts are essential for the inclusion of pharmaceuticals in currently regulatory actions as substances of priority concern that may constitute a risk for non-target species. This is the case of the European surface water Watch List, where several pharmaceuticals have been included, mostly azole compounds and antibiotics, but also neuroactive compounds such as antidepressant venlafaxine and its metabolite O-desmethylvenlafaxine (European Commission, 2022). Notwithstanding, for many neuroactive compounds, information on occurrence and effects is still lacking, particularly in brackish and marine systems (e.g. clozapine, mianserin, clobazam, propofol), and further environmental screening is needed to pinpoint potentially threatening compounds.

Aiming at filling the knowledge gap concerning the occurrence and bioaccumulation of neuroactive pharmaceuticals in estuarine environments, in Chapter 3, a multi-system, multi-taxa, multi-tissue and multiple screening of neuroactive compounds approach was used. Out of 33 neuroactive compounds analysed, 28 were detected in estuarine surface waters, in complex mixtures of at least 10 and up to 26 different compounds. All seven therapeutic classes including frequently detected psychostimulants, antidepressants, opioids and anxiolytics were detected, with individual compounds reaching thousands of ng/L. Despite slight variations in occurrence and concentrations among the four estuaries considered, results evidence the ubiquity and diversity of neuroactive residues in surface waters, occurring in both highly and slightly urbanized systems. Moreover, results revealed unexpected higher concentrations in the two less impacted systems, which may result from higher loads related to sampling season (summer) in combination with lower percentages of dwellings served by wastewater treatment in those systems, possibly contributing to higher and more unpredictable pharmaceutical inputs (Fork et al., 2021; Pereira et al., 2016). Thus, screening for pharmaceuticals in *a priori* considered low impacted areas should not be disregarded. Notably, 15 of the 28 neuroactive compounds were detected at concentrations above 10 ng/L, which is the action limit considered as of potential environmental risk under the marketing authorisation process, and above which studies on environmental fate and effects are required (EMA, 2006). Seven of these 15 compounds were also signalled as of priority concern in Chapter 2. These high and ever-increasing environmental concentrations may be threatening to fish, pointing to the need for prioritization, monitoring and inclusion of neuroactive compounds in future studies.

The bioaccumulation of 13 neuroactive pharmaceuticals was observed among three tissues (brain, liver and muscle) of 7 different fish species. Bioaccumulation was pervasive and critical bioaccumulation patterns were observed. Firstly, neuroactive pharmaceuticals were detected at higher frequency and at higher summed concentrations in the brain, followed by the liver and muscle. This pattern occurred among all species and was consistent amongst the four estuaries sampled. This outcome is somehow expected considering the mode of action of neuroactive pharmaceuticals, whose main targets are located in the brain (Miller et al., 2018; van der Oost et al., 2003), and the bioconcentration pattern observed for neuroactive compounds in laboratory trials (Chapter 2). Likewise, the same pattern has been previously reported in laboratory trials of fish exposed to wastewater effluents and in field studies (e.g. Brooks et al., 2005; Grabicova et al., 2014; Liu et al., 2018), yet previous studies were limited to a reduced number of compounds. Noteworthy, results confirm how critical tissue selection is within the context of bioaccumulation and toxicity assessments, both in laboratory and field studies, which was also one of the main conclusions presented in Chapter 2. As pointed out in the latter, only a small portion of studies determined bioconcentration and only 17% of bioconcentration factors were determined in fish brain, which might be contributing to the underestimation of bioaccumulation and toxicity potentials of neuroactive pharmaceuticals. Secondly, all seven species accumulated neuroactive drugs in their tissues, revealing a general uptake, although at varying degrees. The hypothesis of increased bioaccumulation in species that spend their whole life inside the estuaries (resident species) in comparison with species that use the estuary as a nursery ground or occasionally for feeding purposes (marine migrants or stragglers) was not confirmed by our results. This could be associated with the fast and generalized uptake of pharmaceuticals (e.g. Liu et al., 2021; Wang and Gardinali, 2013), or with the fact that the specimens sampled were late juveniles/young adults, that spend their first years inside the estuary as do resident species, albeit over their entire life-cycle, resulting in similar bioaccumulation. Future research concerning the influence of life-history strategies and habitat use patterns is of added value, as patterns observed for adult specimens, for instance, may differ. Moreover, no bioaccumulation pattern could be associated with species trophic levels, supporting the generalized uptake and bioaccumulation within all seven species. The dynamics of trophic transfer of pharmaceuticals is still poorly addressed, although some recent studies have described a trophic dilution phenomenon between primary producers and top predators, likely related with higher metabolic capacities in top consumers (e.g. Xie et al., 2017). The benefit of using these estuarine and marine species as sentinels of pharmaceutical contamination in coastal environments is now recognized, albeit certain species bioaccumulated higher levels of pharmaceuticals.

Juveniles of European sea bass *Dicentrarchus labrax* and common sole *Solea solea* accumulated total concentrations up to hundreds of ng/g, while European flounder *Platichthys flesus*, Lusitanian toadfish *Halobatrachus didactylus* and gilthead sea bream *Sparus aurata* reached tens of ng/g, whilst in other sea bream species *Diplodus* spp. lower concentrations were found. These different concentration ranges can be associated with varying species-specific traits such as different metabolic rates (Arnot and Gobas, 2006), yet scarce information concerning the uptake and accumulation of pharmaceuticals in different fish species exist (Chapter 2), and these inter-species variations need to be integrated in future studies for further resolution.

The prediction of bioaccumulation through neuroactive pharmaceuticals' lipophilicity, as previously revised in Chapter 2, was also analysed in this chapter (Chapter 3) from the field data obtained, and no significant correlation was found, again supporting that the use of lipophilicity alone is not straightforward for predicting neuroactive pharmaceuticals bioaccumulation, and that the use of limiting thresholds under regulatory frameworks need to be carefully and urgently reviewed. Determining internalized concentrations provides an accurate measure of what fish uptake, while reflecting the final result of multiple interactions (e.g. exposure duration and concentrations; but also individual contributions such as metabolic capacity), and ultimately confirming exposure and bioconcentration (McCarty et al., 2011). While measuring medium (water) concentrations is key for validating occurrence and investigating variability patterns of these compounds in natural ecosystems, the use of single grab water samples is also a limited or not fully representative sampling of the complexity of these matrices. Instead, by analysing tissue concentrations, a direct measurement of highly or frequently accumulated compounds (e.g. topiramate, venlafaxine or risperidone, in Chapter 3) can be obtained, and in some cases, it can even help signal higher risk compounds that are not detected in the medium but bioaccumulated in multiple fish species, such as risperidone (Chapter 3; Fick et al., 2010; Grabicova et al., 2017). Measuring tissue concentrations within the scope of toxicity assessments is paramount for understanding exposure effects, since it is the internalized concentration that will dictate a pharmacological and ultimately a toxicological response (Huggett et al., 2003; Sijm and Hermens, 2005) and it seems fundamental for filling the knowledge gap concerning exposure of wildlife to pharmaceuticals and revealing bioaccumulation patterns in the estuarine environment (Fonseca and Reis-Santos, 2019; Gaw et al., 2014). It is yet unclear for most compounds, however, whether the concentrations detected in biota would be causing adverse effects in these specimens due to the scarcity of studies where both concentrations and toxicological endpoints were measured (Chapter 2), which is integral in defining threshold safety levels for fish in future regulatory and monitoring schemes.

While in Chapter 3 it becomes indisputable that estuarine and marine fish species are subjected to the inevitable encounter with multiple neuroactive pharmaceuticals, resulting in their uptake and bioaccumulation, the impacts of such exposure are still seldom explored, particularly concerning species from brackish and marine environments. In this context, Chapter 4 and 5 consist of experimental trials designed to study affected mechanisms, following exposure to various pharmaceuticals with different modes of action, in different fish species, both integrating multi-biomarker responses at sub-individual and individual levels.

The work developed in Chapter 4 evidenced how fluoxetine affects fish antioxidant defences, biotransformation processes, neurotransmission and behavioural responses of an estuarine species, the common goby *Pomatoschistus microps*. Despite being one of the most studied neuroactive pharmaceuticals, fluoxetine has scarcely been investigated in estuarine and marine species, although its presence in estuarine and coastal waters has been frequently described (Chapter 2; Madikizela et al., 2020; Mezzelani et al., 2018). Fluoxetine toxicity is linked to reactive oxygen species (ROS) production in fish hepatocytes, yet *in vivo* responses vary within the few studies available, with both induction and inhibition of antioxidant defences reported (e.g. Cunha et al., 2016; Ding et al., 2016; Pan et al., 2018). Here, evidence of significant inhibition of antioxidant enzyme catalase with increasing concentrations (mg/L range at 96h) and slight increased or reduced activity (at higher concentrations, 1h) were observed, revealing the complex response of antioxidant mechanisms. On the other hand, biotransformation processes (GST and EROD) were equally modelled by fluoxetine at environmental concentrations, in line with its known influence on biotransformation enzymes (e.g. Kreke and Dietrich, 2008; Laville et al., 2004). A critical hormetic pattern of enzyme induction at low concentrations followed by inhibition at higher concentrations was found for both enzymes. Bell-shaped responses are a common, well-established response to environmental stressors and essential in toxicology, pharmacology and risk assessment, by describing dose-response relations at low doses (Calabrese, 2010), which is crucial for studying the effects of contaminants, like in the case of pharmaceuticals occurring at low µg/L range. The choice of concentrations can, therefore, influence results' interpretation, as previously pointed out by Lopes et al., (2020), concerning the opposite effects of antidepressants observed in fish and crustaceans in distinct concentration ranges tested. Therefore, studies at environmentally relevant concentrations and beyond, like in this chapter, using multiple concentrations, are crucial for studying dose-response relations and potential hormetic effects of pharmaceuticals, as observed for biotransformation enzymes. No oxidative damage to lipids or DNA was detected in *P. microps*, differing from previous studies in which freshwater fish species showed increased lipid peroxidation (Chen et al., 2018; Ding et

al., 2016). First screening for DNA damage in fish was presented here, although decreased or no DNA damage has also been reported in invertebrate species (e.g. Franzellitti et al., 2015; Magni et al., 2017; Maranho et al., 2014). Moreover, contrary to our results, a significant decrease of fish locomotor activity and feeding rates has been observed in other fish species at the same range of concentrations (ng/L – µg/L) (e.g. Meijide et al., 2018; Saaristo et al., 2018; Weinberger and Klaper, 2014). In sum, exposure to environmentally relevant concentrations did not result in overt oxidative damage or behavioural effects in *P. microps*, revealing somehow an inherent tolerance of this species comparing to previous studies in freshwater species, likely a result of adaption to naturally variable estuarine systems (e.g. Elliott and Quintino, 2007). However, exposure to higher concentrations revealed the collapse of acetylcholinesterase activity (AChE) and of fish activity and feeding performances (the number of feeding and active fish was reduced, and fish were less active and showed increased movement delay). The activity of AChE is a biomarker of neurotransmission in fish and invertebrates, of common use in environmental contamination studies (van der Oost et al., 2003). While fluoxetine modulation of serotonin levels is known to occur in fish (e.g. Mennigen et al., 2011), in line with its mode of action, recently the effects towards cholinergic system have been disclosed through the analysis of AChE activity. An increasing trend of activity at environmentally relevant concentrations was reported (e.g. Chen et al., 2018; Pan et al., 2018) yet inhibition of cholinesterases' activity in fish was also previously reported in fish embryos at the ug/L range (de Farias et al., 2019), and now in *P. microps* at higher concentrations (mg/L). Notably, the inhibition of AChE activity was concomitant and highly correlated to the alterations in fish locomotor behaviours (movement delay and activity). While the complexity of the serotonergic system and its relation with other neurotransmitter systems are not quite resolved in fish (Kreke and Dietrich, 2008), the hypothesis of fluoxetine modulation of fish behaviour through changes in the cholinergic system observed here and previously suggested (de Farias et al., 2019; Winder et al., 2012) warrants further investigation. Notwithstanding, these results reveal that fluoxetine might have the potential to influence individual survival and fitness, by compromising important behaviours as feeding and locomotion, that can ultimately lead to changes in population dynamics (e.g. Brodin et al., 2014). Overall, studies of exposure to environmentally relevant concentrations provide key and relevant responses, whereas exposure to higher concentrations are critical in future exposure scenarios and to investigate affected mechanisms and reveal endpoints that should be further investigated.

The ubiquity of pharmaceuticals in the aquatic environment was evident in the previous chapters (Chapters 2 and 3), and albeit some variation was observed within ecosystems, it is

clear that wild fish are continuously exposed to these compounds. Therefore, chronic exposure studies are a valuable representation of environmental conditions, and a key way forward to understanding the impacts of pharmaceuticals in the environment. Chapter 5 revealed different patterns of chronic toxicity in juvenile meagre *Argyrosomus regius* among three pharmaceuticals with different modes of action (MOA): the antidepressant fluoxetine, the non-steroidal anti-inflammatory diclofenac and the antihypertensive propranolol. All three compounds are among the most used and prescribed drugs worldwide and are frequently detected in aquatic environments (e.g. aus der Beek et al., 2016; Wilkinson et al., 2022), though its effects on fish are seldom explored within the chronic exposure context. Chiefly, the results from Chapter 5 revealed differently affected mechanisms among pharmaceuticals, likely associated with higher internal bioconcentration among compounds and different MOA. Extensive bioconcentration of the antidepressant fluoxetine occurred following chronic exposure at concentrations similar to those measured in estuarine waters (hundreds of ng/L, Chapter 3) and at 3 µg/L, more than 10 times lower than maximum reported concentrations in surface waters (Chapter 2). At the highest concentration, chronic exposure resulted in decreased growth, triggered antioxidant defences, inhibition of biotransformation enzymes and increased liver oxidative damage in lipids and DNA. For the first time, chronic exposure to fluoxetine was shown to significantly decrease juvenile fish growth in a brackish-marine species, though in line with some previous studies in freshwater fish species (e.g. Mennigen et al., 2010; Pelli and Connaughton, 2015). Whether growth rate reduction is linked to biochemical alterations, such as serotonin-mediated appetite suppression, or to altered behaviours (e.g. decreased feeding rate as observed in Chapter 4) is yet to be resolved (McDonald, 2017). Interestingly, energy metabolism biomarkers were not significantly altered, although energy consumption was negatively correlated with fish morphometrics (length and weight), and previous studies have reported altered metabolic parameters following chronic exposure in freshwater species (e.g. Mennigen et al., 2010; Mishra et al., 2017). The implications of fluoxetine on fish growth at environmentally relevant concentrations are critical and its link to energy demands warrant further research.

As in the acute exposure trial with *P. microps* (Chapter 4), the same mechanisms were affected by fluoxetine exposure, i.e., altered antioxidant enzymes activities and inhibited biotransformation activities. However, *A. regius* juveniles showed enhanced liver oxidative stress (lipid peroxidation and DNA damage) following chronic exposure at lower environmentally relevant concentrations. While lipid peroxidation was previously associated with fluoxetine exposure (Chen et al., 2018; Ding et al., 2016), genotoxic effects in the liver were firstly evaluated here (Chapter 4 and 5), despite only significant ensuing chronic exposure (Chapter 5), revealing

the potentially hazard of long-term exposure to fluoxetine at environmentally relevant concentrations. As observed in Chapter 4, acetylcholinesterase activity was also not significantly affected at environmentally relevant concentrations in *A. regius*. However, on a preliminary test run with *A. regius* at 15 µg/L (ca. 5 times higher than the concentration used), behavioural distress signs concerning swimming and feeding were observed within 48h, revealing a much sensitive response of *A. regius* to fluoxetine comparing to *P. microps* (that did not exhibit behavioural changes up to 96h and at concentrations as high as 100 µg/L). Whether differences in responses observed among the two fish species are linked to species-specific traits, life-stages differences (*P. microps* adults vs *A. regius* juveniles) or variations in specimens' origin (field collected vs aquaculture), needs to be further explored, yet it corroborates with the substantial inter-studies variability observed in Chapter 2.

The antihypertensive propranolol showed intermediate toxicity and lower bioconcentration than fluoxetine, albeit increased muscle DNA damage and reduced aerobic metabolism were observed. In line with previous studies, propranolol metabolism appears to be more efficient than that of fluoxetine in fish hepatocytes (e.g. Connors et al. (2013)), likely contributing to lower tissue concentrations observed. Moreover, exposure to propranolol did not cause alterations to antioxidant, biotransformation or acetylcholinesterase enzymes as observed for freshwater species (Bartram et al., 2011; Pereira et al., 2018). Interestingly, propranolol caused a shift to anaerobic metabolism in muscle tissues, likely associated with increased oxidative stress, as revealed by concomitant increased DNA damage in fish muscle. While previous studies report propranolol interference in metabolic processes linked to its binding to hepatic β-adrenoceptors, in freshwater species (Fabbri et al., 1998; Gesto et al., 2014), the impact of propranolol in estuarine and marine fish metabolism is still fairly unknown.

Anti-inflammatory diclofenac did not accumulate in fish muscle tissues, contrary to what would have been assumed based on the compound's lipophilicity, since it has the highest $\log K_{ow}$ of all three compounds tested, supporting the results from Chapters 2 and 3, albeit in this case not just considering neuroactive compounds. Diclofenac, akin to propranolol, did not cause alterations to antioxidant or biotransformation enzymes, nor neurotoxic effects, generally in agreement with its low toxicity to fish (e.g. Ghelfi et al., 2016; Maulvault et al., 2018; Schwarz et al., 2017). However, it affected fish metabolism by increasing energy consumption and reducing the fish net energy budget at both concentrations tested, possibly as a result of energy expenditure required for extensive diclofenac metabolism. Though the increased energy demand was not sufficient to decrease energy reserves (lipids, proteins or carbohydrates) after 30 days, effects at longer exposure times cannot be disregarded, including decreased juvenile

growth rates (which were slightly, though not significantly, reduced). Overall, differences in bioconcentration potentials seem to support increased toxicity among pharmaceuticals, whereas, as expected, pharmaceuticals with different MOA evidenced different toxicity and effects, emphasizing the need to further investigate the mechanisms underlying pharmaceuticals' toxicity in fish, which are, for many compounds, not yet fully investigated. Here, the results presented in Chapter 4 and 5 highlight that approaches combining sub-individual and individual responses are key for ecologically relevant assessments of pharmaceuticals' toxicity.

Ultimately, this thesis presents important results of ecotoxicological trials under controlled conditions, that are crucial for studying the applicability of biomarker measurements and to unravel specific targeted mechanisms in pharmaceutical toxicity studies. Furthermore, results reveal the complexity of pharmaceutical pollution in natural environments, that are crucial for prioritization and improving risk assessment of neuroactive pharmaceuticals. The outcomes of this thesis also emphasize knowledge gaps and further research needs concerning the occurrence and exposure effects of neuroactive pharmaceuticals to non-target species. Underlying the high variability of bioconcentration and effects observed among neuroactive pharmaceuticals, the need to further understand toxicokinetics and toxicodynamics of pharmaceuticals is highlighted. In this context, it is essential to address the influence of experimental parameters (e.g. temperature, salinity, pH, exposure time) but also to unravel inter- and intra-species variability (e.g. life-stage, tissue or species-specific traits), including differences inherent to wildlife and laboratory reared specimens. Omics approaches can also be valuable tools by providing new insights concerning toxicity mechanisms or affected pathways (e.g. Zhang et al., 2018) linked to the observed alterations in pharmaceutical biotransformation, oxidative stress, growth and metabolism or behavioural processes. Furthermore, a much-needed inclusion of marine and brackish species in future studies is also evident, still understudied compared to freshwater species. The insufficient environmental data available for multiple neuroactive pharmaceuticals in these transitional ecosystems was also stressed, concerning both the occurrence in surface waters and bioaccumulation in fish, which is typically addressed for a reduced number of pharmaceuticals. Monitoring the environmental occurrence of pharmaceuticals is key to address and prioritize persistent and ubiquitous pharmaceuticals (e.g. compounds exceeding MRC or action limit concentrations) as well as highly or frequently bioaccumulated compounds. Moreover, the use of internalized concentrations is paramount for inter-studies comparison and linking exposure to toxicity effects and should be considered in future studies. The results of such a combined approach would help to understand if internalized concentrations found in wildlife biota extrapolate to adverse effects, that might be threatening these species in their natural

environments and allow for the integration of such compounds in prioritization and mitigation strategies. The importance of chronic studies is also highlighted as different responses can be observed within acute and chronic exposures, which is highly relevant considering the ubiquity and pseudo-persistency of pharmaceuticals in the aquatic environment.

Ultimately, this thesis provides critical insights for the revision of the risk assessment of neuroactive pharmaceutical compounds in aquatic environments, with direct implications on management of water quality, within the scope of environmental directives (e.g. the European surface water Watch List, as per the Water Framework Directive, European Commission, 2000). Moreover, results emphasize the urgency for innovative strategies, combining science communication for public engagement and implementation of technological solutions (e.g. on the wastewater treatment side), to significantly reduce the discharge and the environmental risk of these emerging contaminants in the aquatic environment, from rivers to the sea.

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APPENDICES

APPENDIX 1 - Supplementary material of Chapter 2

APPENDIX 2 - Supplementary material of Chapter 3

APPENDIX 3 - Supplementary material of Chapter 5

APPENDIX 1

Supplementary material of Chapter 2

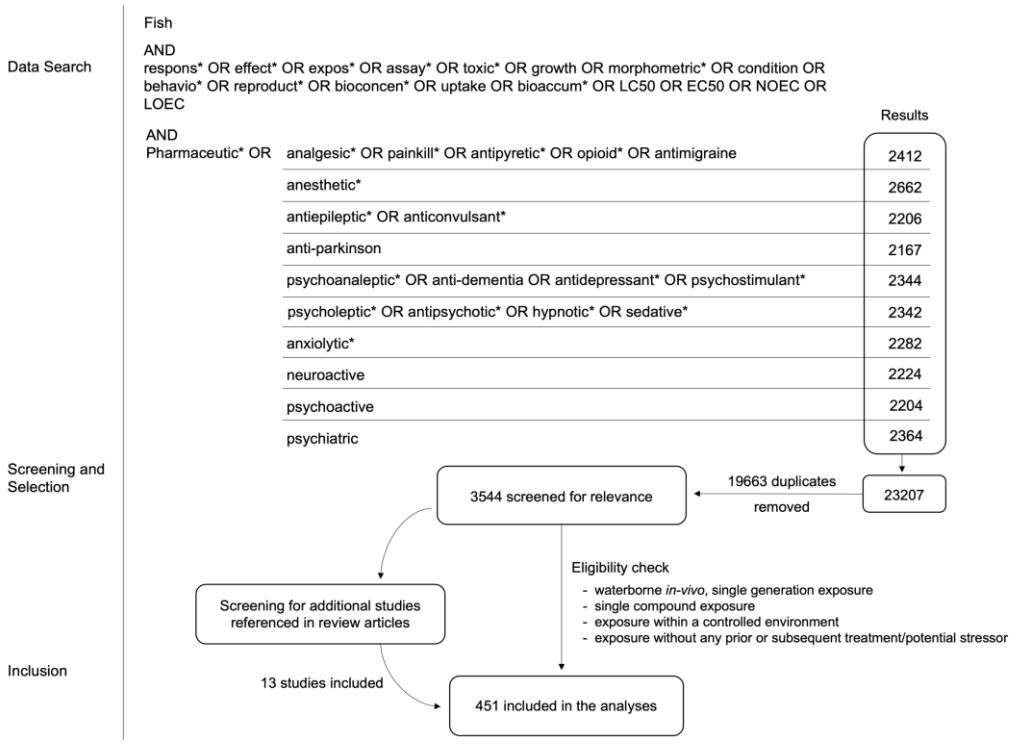

Figure A 1.1. Full details on the systematic literature search steps (data search, screening, selection and inclusion), terms used and results obtained.

Table A 1.1. Partial reproduction of the list of compounds included in the Anatomical Therapeutic Chemical (ATC) classification system from WHO (World Health Organization), with classification levels within category N (that includes all compounds acting on the nervous system). Full table available at https://doi. org/10.1016/j.scitotenv.2021.152543.

ATC 2nd level	ATC 3rd level	ATC 4th level	ATC 5th level
N01 ANESTHETICS	N01A ANESTHETICS, GENERAL	N01AA Ethers	N01AA01 Diethyl ether
			N01AA02 Vinyl ether
		N01AB Halogenated hydrocarbons	N01AB01 Halothane
			N01AB02 Chloroform
			N01AB04 Enflurane
			N01AB05 Trichloroethylene
			N01AB06 Isoflurane
			N01AB07 Desflurane
			N01AB08 Sevoflurane

Table A 1.2. List of all publications considered in this study.

Year	Authors	Title	Journal	DOI
2020	Hellstrom, G; Brodin, T; Jonsson, M; Olsen, H; Leander, J; Fahlman, J; Fick, J; Klaminder, J	Environmentally relevant concentrations of the common anxiolytic pharmaceutical oxazepam do not have acute effect on spawning behavior in mature male Atlantic salmon (*Salmo salar*) parr	JOURNAL OF APPLIED ICHTHYOLOGY	10.1111/jai.13980
2020	Cerveny, D; Brodin, T; Cisar, P; McCallum, ES; Fick, J	Bioconcentration and behavioral effects of four benzodiazepines and their environmentally relevant mixture in wild fish	SCIENCE OF THE TOTAL ENVIRONMENT	10.1016/j.scitotenv.2019.134780
2020	Vossen, LE; Cerveny, D; Sen Sarma, O; Thornqvist, PO; Jutfelt, F; Fick, J; Brodin, T; Winberg, S	Low concentrations of the benzodiazepine drug oxazepam induce anxiolytic effects in wild-caught but not in laboratory zebrafish	SCIENCE OF THE TOTAL ENVIRONMENT	10.1016/j.scitotenv.2019.134701
2020	Zhao, ZK; Li, G; Xiao, Q; Jiang, HR; Tchivelekete, GM; Shu, XH; Liu, H	Quantification of the influence of drugs on zebrafish larvae swimming kinematics and energetics	PEERJ	10.7717/peerj.8374
2020	Rezaei, M; Moradi, AM; Mortazavi, P; Jamili, S	Effects of chronic exposure to carbamazepine on hematological parameters in *Cyprinus carpio*	IRANIAN JOURNAL OF FISHERIES SCIENCES	10.22092/IJFS.2019.119014
2020	Vossen, LE; Cerveny, D; Osterkrans, M; Thornqvist, PO; Jutfelt, F; Fick, J; Brodin, T; Winberg, S	Chronic Exposure to Oxazepam Pollution Produces Tolerance to Anxiolytic Effects in Zebrafish (*Danio rerio*)	ENVIRONMENTAL SCIENCE & TECHNOLOGY	10.1021/acs.est.9b06052
2020	Duarte, IA; Reis-Santos, P; Novais, SC; Rato, LD; Lemos, MFL; Freitas, A; Pouca, ASV; Barbosa, J; Cabral, HN; Fonseca, VF	Depressed, hypertense and sore: Long-term effects of fluoxetine, propranolol and diclofenac exposure in a top predator fish	SCIENCE OF THE TOTAL ENVIRONMENT	10.1016/j.scitotenv.2020.136564
2020	Vazquez, GR; Da Cuna, RH; Dorelle, LS; Lo Nostro, FL	Immunohistological Biomarkers of Toxicity by a Pharmaceutical Antidepressant in the Freshwater Cichlid Fish *Cichlasoma dimerus* (Teleostei, Cichliformes)	BULLETIN OF ENVIRONMENTAL CONTAMINATION AND TOXICOLOGY	10.1007/s00128-019-02770-3
2020	Barcellos, HHD; Pompermaier, A; Mendonca-Soares, S; Maffi, VC; Fernandes, M; Koakoski, G; Kirsten, K; Baldisserotto, B; Barcellos, LJG	Aripiprazole prevents stress-induced anxiety and social impairment, but impairs antipredatory behavior in zebrafish	PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR	10.1016/j.pbb.2019.172841
2020	Nowakowska, K; Giebultowicz, J; Kamaszewski, M; Adamski, A; Szudrowicz, H; Ostaszewska, T; Solarska-Dzieciolowska, U; Nalecz-Jawecki, G; Wroczynski, P; Drobniewska, A	Acute exposure of zebrafish (*Danio rerio*) larvae to environmental concentrations of selected antidepressants: Bioaccumulation, physiological and histological changes	COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY C-TOXICOLOGY & PHARMACOLOGY	10.1016/j.cbpc.2019.108670

2020	Ikert, H; Craig, PM	Chronic exposure to venlafaxine and increased water temperature reversibly alters microrna in zebrafish gonads (*Danio rerio*)	COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY D-GENOMICS & PROTEOMICS	10.1016/j.cbd.2019.100634
2020	Sivalingam, M; Ogawa, S; Parhar, IS	Mapping of Morphine-Induced OPRM1 Gene Expression Pattern in the Adult Zebrafish Brain	FRONTIERS IN NEUROANATOMY	10.3389/fnana.2020.00005
2020	dos Santos, PRD; Costa, MJ; Dos Santos, ACA; Silva-Zacarin, ECM; Nunes, B	Neurotoxic and respiratory effects of human use drugs on a Neotropical fish species, *Phalloceros harpagos*	COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY C-TOXICOLOGY & PHARMACOLOGY	10.1016/j.cbpc.2019.108683
2020	Godoi, FGA; Munoz-Penuela, M; Gomes, ADO; Tolussi, CE; Brambila-Souza, G; Branco, GS; Lo Nostro, FL; Moreira, RG	Endocrine disruptive action of diclofenac and caffeine on *Astyanax altiparanae* males (Teleostei: Characiformes: Characidae)	COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY C-TOXICOLOGY & PHARMACOLOGY	10.1016/j.cbpc.2020.108720
2020	He, RP; Lei, B; Su, YP; Wang, AL; Cui, K; Shi, XK; Chen, XM	Effectiveness of eugenol as an anesthetic for adult spotted sea bass (*Lateolabrax maculatus*)	AQUACULTURE	10.1016/j.aquaculture.2020.735180
2020	Zindler, F; Tisler, S; Loerracher, AK; Zwiener, C; Braunbeck, T	Norfluoxetine Is the Only Metabolite of Fluoxetine in Zebrafish (*Danio rerio*) Embryos That Accumulates at Environmentally Relevant Exposure Scenarios	ENVIRONMENTAL SCIENCE & TECHNOLOGY	10.1021/acs.est.9b07618
2020	Cedron, VP; Weiner, AMJ; Vera, M; Sanchez, L	Acetaminophen affects the survivor, pigmentation and development of craniofacial structures in zebrafish (*Danio rerio*) embryos	BIOCHEMICAL PHARMACOLOGY	10.1016/j.bcp.2020.113816
2020	Alfonso, S; Sadoul, B; Cousin, X; Begout, ML	Spatial distribution and activity patterns as welfare indicators in response to water quality changes in European sea bass, *Dicentrarchus labrax*	APPLIED ANIMAL BEHAVIOUR SCIENCE	10.1016/j.applanim.2020.104974
2020	Plhalova, L; Sehonova, P; Blahova, J; Doubkova, V; Tichy, F; Faggio, C; Berankova, P; Svobodova, Z	Evaluation of Tramadol Hydrochloride Toxicity to Juvenile Zebrafish-Morphological, Antioxidant and Histological Responses	APPLIED SCIENCES-BASEL	10.3390/app10072349
2020	Ferreira, AL; Silva, WDE; Neves, LD; Ferreira, NS; Takata, R; Luz, RK	Benzocaine and menthol as anesthetics for the African cichlid *Aulonocara nyassae*	AQUACULTURE INTERNATIONAL	10.1007/s10499-020-00561-w
2020	Sousa, B; Nunes, B	Reliability of behavioral test with fish: How neurotransmitters may exert neuromodulatory effects and alter the biological responses to neuroactive agents	SCIENCE OF THE TOTAL ENVIRONMENT	10.1016/j.scitotenv.2020.139372
2020	Dean, R; Duperreault, E; Newton, D; Krook, J; Ingraham, E; Gallup, J; Franczak, BC; Hamilton, TJ	Opposing effects of acute and repeated nicotine exposure on boldness in zebrafish	SCIENTIFIC REPORTS	10.1038/s41598-020-65382-6
2020	Dong, HB; Wang, WH; Duan, YF; Li, H; Liu, QS; Sun, YX; Zhang, JS	Transcriptomic analysis of juvenile Chinese sea bass (*Lateolabrax maculatus*) anesthetized by MS-222 (tricaine methanesulfonate) and eugenol	FISH PHYSIOLOGY AND BIOCHEMISTRY	10.1007/s10695-019-00755-x

2020	McCord, CL; Whiteley, E; Liang, J; Trejo, C; Caputo, R; Itehua, E; Hasan, H; Hernandez, S; Jagnandan, K; Fudge, D	Concentration effects of three common fish anesthetics on Pacific hagfish (*Eptatretus stoutii*)	FISH PHYSIOLOGY AND BIOCHEMISTRY	10.1007/s10695-020-00761-4
2020	Nascimento, HD; Crispim, BD; Francisco, LFV; Merey, FM; Kummrow, F; Viana, LF; Inoue, LAKA; Barufatti, A	Genotoxicity evaluation of three anesthetics commonly employed in aquaculture using *Oreochromis niloticus* and *Astyanax lacustris*	AQUACULTURE REPORTS	10.1016/j.aqrep.2020.100357
2020	Hubena, P; Horky, P; Grabic, R; Grabicova, K; Slavik, O; Randak, T	Environmentally relevant levels of four psychoactive compounds vary in their effects on freshwater fish condition: a brain concentration evidence approach	PEERJ	10.7717/peerj.9356
2020	Rosa, LV; Costa, FV; Canzian, J; Borba, JV; Quadros, VA; Rosemberg, DB	Three- and bi-dimensional analyses of the shoaling behavior in zebrafish: Influence of modulators of anxiety-like responses	PROGRESS IN NEUROPSYCHOPHARMACOLOGY & BIOLOGICAL PSYCHIATRY	10.1016/j.pnpbp.2020.109957
2020	Yu, N; Cao, XH; Wang, YJ; Kuang, SW; Hu, JB; Yang, Y; Xu, SL; Zhang, M; Sun, YB; Gu, WW; Yan, XJ	Reduced stress responses by MS-222 in juvenile silver pomfret (*Pampus argenteus*)	JOURNAL OF THE WORLD AQUACULTURE SOCIETY	10.1111/jwas.12725
2020	Soliman, HAM; Sayed, AEH	Poikilocytosis and tissue damage as negative impacts of tramadol on juvenile of Tilapia (*Oreochromis niloticus*)	ENVIRONMENTAL TOXICOLOGY AND PHARMACOLOGY	10.1016/j.etap.2020.103383
2020	Hoglund, E; Overli, O; Atland, A	Assaying waterborne psychoactive drugs by the response to naturalistic predator cues in the stickleback (*Gasterosteus aculeatus*)	SCIENCE OF THE TOTAL ENVIRONMENT	10.1016/j.scitotenv.2020.140257
2020	Santos, LHMLM; Maulvault, AL; Jaen-Gil, A; Marques, A; Barcelo, D; Rodriguez-Mozaz, S	Insights on the metabolization of the antidepressant venlafaxine by meagre (*Argyrosomus regius*) using a combined target and suspect screening approach	SCIENCE OF THE TOTAL ENVIRONMENT	10.1016/j.scitotenv.2020.140226
2019	Faria, M; Prats, E; Novoa-Luna, KA; Bedrossiantz, J; Gomez-Canela, C; Gomez-Olivan, LM; Raldua, D	Development of a vibrational startle response assay for screening environmental pollutants and drugs impairing predator avoidance	SCIENCE OF THE TOTAL ENVIRONMENT	10.1016/j.scitotenv.2018.08.421
2019	Fursdon, JB; Martin, JM; Bertram, MG; Lehtonen, TK; Wong, BBM	The pharmaceutical pollutant fluoxetine alters reproductive behaviour in a fish independent of predation risk	SCIENCE OF THE TOTAL ENVIRONMENT	10.1016/j.scitotenv.2018.09.046
2019	Martin, JM; Bertram, MG; Saaristo, M; Ecker, TE; Hannington, SL; Tanner, JL; Michelangeli, M; O'Bryan, MK; Wong, BBM	Impact of the widespread pharmaceutical pollutant fluoxetine on behaviour and sperm traits in a freshwater fish	SCIENCE OF THE TOTAL ENVIRONMENT	10.1016/j.scitotenv.2018.09.294
2019	Mishra, P; Gong, ZY; Kelly, BC	Assessing pH-dependent toxicity of fluoxetine in embryonic zebrafish using mass spectrometry-based metabolomics	SCIENCE OF THE TOTAL ENVIRONMENT	10.1016/j.scitotenv.2018.09.364
2019	de Farias, NO; Oliveira, R; Sousa-Moura, D; de Oliveira, RCS; Rodrigues, MAC; Andrade, TS; Domingues, I;	Exposure to low concentration of fluoxetine affects development, behaviour and acetylcholinesterase activity of zebrafish embryos	COMPARATIVE BIOCHEMISTRY AND	10.1016/j.cbpc.2018.08.009

	Camargo, NS; Muehlmann, LA; Grisolia, CK		PHYSIOLOGY C-TOXICOLOGY & PHARMACOLOGY	
2019	Thore, ESJ; Steenaerts, L; Philippe, C; Gregoir, AF; Brendonck, L; Pinceel, T	Improving the reliability and ecological validity of pharmaceutical risk assessment: Turquoise killifish (*Nothobranchius furzeri*) as a model in behavioral ecotoxicology	ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY	10.1002/etc.4301
2019	Saaristo, M; Lagesson, A; Bertram, MG; Fick, J; Klaminder, J; Johnstone, CP; Wong, BBM; Brodin, T	Behavioural effects of psychoactive pharmaceutical exposure on European perch (*Perca fluviatilis*) in a multi-stressor environment	SCIENCE OF THE TOTAL ENVIRONMENT	10.1016/j.scitotenv.2018.11.228
2019	Uehara, SA; Andrade, DR; Takata, R; Gomes, AV; Vidal, MV	The effectiveness of tricaine, benzocaine, clove oil, and menthol as anesthetics for lambari-bocarra *Oligosarcus argenteus*	AQUACULTURE	10.1016/j.aquaculture.2018.12.054
2019	Kenter, LW; Gunn, MA; Berlinsky, DL	Transport Stress Mitigation and the Effects of Preanesthesia on Striped Bass	NORTH AMERICAN JOURNAL OF AQUACULTURE	10.1002/naaq.10072
2019	Parvathy, U; Kumar, KS; Binsi, PK; Nambiar, L; Ninan, G; Zynudheen, AA	Effect of Anaesthetics, Temperature and Aeration in Live Transportation of Tilapia (*Oreochromis mossambicus*) (Peters, 1852)	FISHERY TECHNOLOGY	http://drs.cift.res.in/handle/123456789/4229
2019	Ribeiro, PAP; Hoyos, DCD; de Oliveira, CG; Della Flora, MAL; Luz, RK	Eugenol and benzocaine as anesthetics for Lophiosilurus alexandri juvenile, a freshwater carnivorous catfish	AQUACULTURE INTERNATIONAL	10.1007/s10499-018-0326-3
2019	McCallum, ES; Sundelin, A; Fick, J; Alanara, A; Klaminder, J; Hellstrom, G; Brodin, T	Investigating tissue bioconcentration and the behavioural effects of two pharmaceutical pollutants on sea trout (*Salmo trutta*) in the laboratory and field	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2018.11.028
2019	Le, QJ; Hu, JB; Cao, XH; Kuang, SW; Zhang, M; Yu, N; Zheng, HK; Wang, YJ; Liu, HW; Yan, XJ	Transcriptomic and cortisol analysis reveals differences in stress alleviation by different methods of anesthesia in Crucian carp (*Carassius auratus*)	FISH & SHELLFISH IMMUNOLOGY	10.1016/j.fsi.2018.10.061
2019	Devi, AA; Kamilya, D	Efficacy and effects of clove oil and MS-222 on the immune-biochemical responses of juvenile rohu *Labeo rohita*	AQUACULTURE RESEARCH	10.1111/are.13980
2019	Maulvault, AL; Camacho, C; Barbosa, V; Alves, R; Anacleto, P; Pousao-Ferreira, P; Rosa, R; Marques, A; Diniz, MS	Living in a multi-stressors environment: An integrated biomarker approach to assess the ecotoxicological response of meagre (*Argyrosomus regius*) to venlafaxine, warming and acidification	ENVIRONMENTAL RESEARCH	10.1016/j.envres.2018.10.021
2019	Gasca-Perez, E; Galar-Martinez, M; Garcia-Medina, S; Perez-Coyotl, IA; Ruiz-Lara, K; Cano-Viveros, S; Borja, RPP; Gomez-Olivan, LM	Short-term exposure to carbamazepine causes oxidative stress on common carp (*Cyprinus carpio*)	ENVIRONMENTAL TOXICOLOGY AND PHARMACOLOGY	10.1016/j.etap.2018.12.017
2019	Hope, BV; Hamilton, TJ; Hurd, PL	Submerged plus maze: A novel test for studying anxiety-like behaviour in fish	BEHAVIOURAL BRAIN RESEARCH	10.1016/j.bbr.2018.12.012

2019	Gomes, TB; Sales, SF; Saint'Pierre, TD; Correia, FV; Hauser-Davis, RA; Saggioro, EM	Sublethal psychotropic pharmaceutical effects on the model organism *Danio rerio*: Oxidative stress and metal dishomeostasis	ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY	10.1016/j.ecoenv.2019.01.041
2019	Martinez, R; Vera-Chang, MN; Haddad, M; Zon, J; Navarro-Martin, L; Trudeau, VL; Mennigen, JA	Developmental fluoxetine exposure in zebrafish reduces offspring basal cortisol concentration via life stage-dependent maternal transmission	PLOS ONE	10.1371/journal.pone.0212577
2019	Lomba, L; Ribate, MP; Zuriaga, E; Garcia, CB; Giner, B	Acute and subacute effects of drugs in embryos of *Danio rerio*. QSAR grouping and modelling	ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY	10.1016/j.ecoenv.2019.01.081
2019	Zhou, SB; Chen, QQ; Di Paolo, C; Shao, Y; Hollert, H; Seiler, TB	Behavioral profile alterations in zebrafish larvae exposed to environmentally relevant concentrations of eight priority pharmaceuticals	SCIENCE OF THE TOTAL ENVIRONMENT	10.1016/j.scitotenv.2019.01.300
2019	Tanoue, R; Margiotta-Casaluci, L; Huerta, B; Runnalls, TJ; Eguchi, A; Nomiyama, K; Kunisue, T; Tanabe, S; Sumpter, JP	Protecting the environment from psychoactive drugs: Problems for regulators illustrated by the possible effects of tramadol on fish behaviour	SCIENCE OF THE TOTAL ENVIRONMENT	10.1016/j.scitotenv.2019.02.090
2019	Sundin, J; Jutfelt, F; Thorlacius, M; Fick, J; Brodin, T	Behavioural alterations induced by the anxiolytic pollutant oxazepam are reversible after depuration in a freshwater fish	SCIENCE OF THE TOTAL ENVIRONMENT	10.1016/j.scitotenv.2019.02.049
2019	Franco, MB; Andrade, TS; Sousa-Moura, D; da Silva, ML; Ferraz, IBM; Camargo, NS; Domingues, I; Oliveira, R; Grisolia, CK	Exposure to dilute concentrations of bupropion affects zebrafish early life stages	CHEMOSPHERE	10.1016/j.chemosphere.2019.01.141
2019	Norton, WHJ; Gutierrez, HC	The three-spined stickleback as a model for behavioural neuroscience	PLOS ONE	10.1371/journal.pone.0213320
2019	Wang, WH; Dong, HB; Sun, YX; Cao, M; Duan, YF; Li, H; Liu, QS; Gu, QH; Zhang, JS	The efficacy of eugenol and tricaine methanesulphonate as anaesthetics for juvenile Chinese sea bass (*Lateolabrax maculatus*) during simulated transport	JOURNAL OF APPLIED ICHTHYOLOGY	10.1111/jai.13844
2019	Huang, IJ; Sirotkin, HI; McElroy, AE	Varying the exposure period and duration of neuroactive pharmaceuticals and their metabolites modulates effects on the visual motor response in zebrafish (*Danio rerio*) larvae	NEUROTOXICOLOGY AND TERATOLOGY	10.1016/j.ntt.2019.01.006
2019	Alsop, D; Wilson, JY	Waterborne pharmaceutical uptake and toxicity is modified by ph and dissolved organic carbon in zebrafish	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2019.02.008
2019	Qu, H; Ma, RX; Wang, B; Yang, J; Duan, L; Yu, G	Enantiospecific toxicity, distribution and bioaccumulation of chiral antidepressant venlafaxine and its metabolite in loach (*Misgurnus anguillicaudatus*) co-exposed to microplastic and the drugs	JOURNAL OF HAZARDOUS MATERIALS	10.1016/j.jhazmat.2018.04.041
2019	Neri, D; Ruberto, T; Mwaffo, V; Bartolini, T; Porfiri, M	Social environment modulates anxiogenic effects of caffeine in zebrafish	BEHAVIOURAL PHARMACOLOGY	10.1097/FBP.0000000000000415

2019	McCallum, ES; Lindberg, RH; Andersson, PL; Brodin, T	Stability and uptake of methylphenidate and ritalinic acid in nine-spine stickleback (*Pungitius pungitius*) and water louse (*Asellus aquaticus*)	ENVIRONMENTAL SCIENCE AND POLLUTION RESEARCH	10.1007/s11356-019-04557-9
2019	Ziarrusta, H; Ribbenstedt, A; Mijangos, L; Picart-Armada, S; Perera-Lluna, A; Prieto, A; Izagirre, U; Benskin, JP; Olivares, M; Zuloaga, O; Etxebarria, N	Amitriptyline at an Environmentally Relevant Concentration Alters the Profile of Metabolites Beyond Monoamines in Gilt-Head Bream	ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY	10.1002/etc.4381
2019	Faria, M; Bedrossiantz, J; Prats, E; Garcia, XR; Gomez-Canela, C; Pina, B; Raldua, D	Deciphering the mode of action of pollutants impairing the fish larvae escape response with the vibrational startle response assay	SCIENCE OF THE TOTAL ENVIRONMENT	10.1016/j.scitotenv.2019.03.469
2019	Pohl, J; Ahrens, L; Carlsson, G; Golovko, O; Norrgren, L; Weiss, J; Orn, S	Embryotoxicity of ozonated diclofenac, carbamazepine, and oxazepam in zebrafish (*Danio rerio*)	CHEMOSPHERE	10.1016/j.chemosphere.2019.03.034
2019	Douda, K; Zhao, SR; Vodakova, B; Horky, P; Grabicova, K; Bazkova, K; Grabic, R; Slavik, O; Randak, T	Host-parasite interaction as a toxicity test endpoint using asymmetrical exposures	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2019.04.006
2019	Martin, JM; Bertram, MG; Saaristo, M; Fursdon, JB; Hanninton, SL; Brooks, BW; Burket, SR; Mole, RA; Deal, NDS; Wong, BBM	Antidepressants in Surface Waters: Fluoxetine Influences Mosquitofish Anxiety-Related Behavior at Environmentally Relevant Levels	ENVIRONMENTAL SCIENCE & TECHNOLOGY	10.1021/acs.est.9b00944
2019	Jerez-Cepa, I; Fernandez-Castro, M; O'Neill, TJD; Martos-Sitcha, JA; Martinez-Rodriguez, G; Mancera, JM; Ruiz-Jarabo, I	Transport and Recovery of Gilthead Seabream (*Sparus aurata* L.) Sedated With Clove Oil and MS-222: Effects on Stress Axis Regulation and Intermediary Metabolism	FRONTIERS IN PHYSIOLOGY	10.3389/fphys.2019.00612
2019	Duarte, IA; Pais, MP; Reis-Santos, P; Cabral, HN; Fonseca, VF	Biomarker and behavioural responses of an estuarine fish following acute exposure to fluoxetine	MARINE ENVIRONMENTAL RESEARCH	10.1016/j.marenvres.2019.04.002
2019	Sousa-Moura, D; Matsubara, EY; Ferraz, IBM; de Oliveira, R; Szlachetka, IO; da Silva, SW; Camargo, NS; Rosolen, JM; Grisolia, CK; da Rocha, MCO	Cnts coated charcoal as a hybrid composite material: Adsorption of fluoxetine probed by zebrafish embryos and its potential for environmental remediation	CHEMOSPHERE	10.1016/j.chemosphere.2019.05.019
2019	Tisler, S; Zindler, F; Freeling, F; Nodler, K; Toelgyesi, L; Braunbeck, T; Zwiener, C	Transformation Products of Fluoxetine Formed by Photodegradation in Water and Biodegradation in Zebrafish Embryos (*Danio rerio*)	ENVIRONMENTAL SCIENCE & TECHNOLOGY	10.1021/acs.est.9b00789
2019	Duarte, T; Fontana, BD; Muller, TE; Bertoncello, KT; Canzian, J; Rosemberg, DB	Nicotine prevents anxiety-like behavioral responses in zebrafish	PROGRESS IN NEUROPSYCHOPHARMACOLOGY & BIOLOGICAL PSYCHIATRY	10.1016/j.pnpbp.2019.109655

2019	Rodrigues, A; Borges, FO; Pissarra, V; Maulvault, AL; Paula, JR; Bispo, R; Rosa, R	First indication of deleterious impacts in white-seabream larvae (*Diplodus sargus*) survival and behaviour following acute venlafaxine exposure	ECOTOXICOLOGY	10.1007/s10646-019-02057-7
2019	Delafkar, K; Sattari, M; Khara, H; Poursaeid, S; Falahatkar, B	Sedative Efficacy of Tobacco Extract, Clove Oil, Tricaine Methanesulfonate, and Ketamine: Effects on Hematological Parameters and Blood Biochemical Profile in Sterlet	NORTH AMERICAN JOURNAL OF AQUACULTURE	10.1002/naaq.10094
2019	Chatakondi, NG; Kelly, AM	Evaluation of a Portable Electrosedation System for Anesthetizing Mature Channel Catfish	NORTH AMERICAN JOURNAL OF AQUACULTURE	10.1002/naaq.10095
2019	Batalhao, IG; Lima, D; Russi, APM; Boscolo, CNP; Silva, DGH; Pereira, TSB; Bainy, ACD; de Almeida, EA	Effects of methylphenidate on the aggressive behavior, serotonin and dopamine levels, and dopamine-related gene transcription in brain of male Nile tilapia (*Oreochromis niloticus*)	FISH PHYSIOLOGY AND BIOCHEMISTRY	10.1007/s10695-019-00645-2
2019	Young, T; Walker, SP; Alfaro, AC; Fletcher, LM; Murray, JS; Lulijwa, R; Symonds, J	Impact of acute handling stress, anaesthesia, and euthanasia on fish plasma biochemistry: implications for veterinary screening and metabolomic sampling	FISH PHYSIOLOGY AND BIOCHEMISTRY	10.1007/s10695-019-00669-8
2019	Aliko, V; Mehmeti, E; Qirjo, M; Faggio, C	Drink and sleep like a fish: goldfish as a behavior model to study pharmaceutical effects in freshwater ecosystems	JOURNAL OF BIOLOGICAL RESEARCH-BOLLETTINO DELLA SOCIETA ITALIANA DI BIOLOGIA SPERIMENTALE	10.4081/jbr.2019.7939
2019	Nielsen, SV; Frausing, M; Henriksen, PG; Beedholm, K; Baatrup, E	The psychoactive drug escitalopram affects foraging behavior in zebrafish (*Danio rerio*)	ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY	10.1002/etc.4474
2019	Klaminder, J; Jonsson, M; Leander, J; Fahlman, J; Brodin, T; Fick, J; Hellstrom, G	Less anxious salmon smolt become easy prey during downstream migration	SCIENCE OF THE TOTAL ENVIRONMENT	10.1016/j.scitotenv.2019.05.488
2019	Perussolo, MC; Guiloski, IC; Lirola, JR; Fockink, DH; Corso, CR; Bozza, DC; Prodocimo, V; Mela, M; Ramos, LP; Cestari, MM; Acco, A; de Assis, HCS	Integrated biomarker response index to assess toxic effects of environmentally relevant concentrations of paracetamol in a neotropical catfish (*Rhamdia quelen*)	ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY	10.1016/j.ecoenv.2019.109438
2019	Alia, AO; Petrunich-Rutherford, ML	Anxiety-like behavior and whole-body cortisol responses to components of energy drinks in zebrafish (*Danio rerio*)	PEERJ	10.7717/peerj.7546
2019	Kalichak, F; Barcellos, HHD; Idalencio, R; Koakoski, G; Soares, SM; Pompermaier, A; Rossini, M; Barcellos, LJG	Persistent and transgenerational effects of risperidone in zebrafish	ENVIRONMENTAL SCIENCE AND POLLUTION RESEARCH	10.1007/s11356-019-05890-9
2019	Xie, ZX; Lu, GH	Interactive Effects of Sertraline and Diphenhydramine on Biochemical and Behavioral Responses in Crucian Carp (*Carassius auratus*)	INTERNATIONAL JOURNAL OF ENVIRONMENTAL RESEARCH AND PUBLIC HEALTH	10.3390/ijerph16173137

2019	Vera-Chang, MN; St-Jacques, AO; Lu, CY; Moon, TW; Trudeau, VL	Fluoxetine Exposure During Sexual Development Disrupts the Stress Axis and Results in Sex- and Time-Dependent Effects on the Exploratory Behavior in Adult Zebrafish *Danio rerio*	FRONTIERS IN NEUROSCIENCE	10.3389/fnins.2019.01015
2019	Sehonova, P; Hodkovicova, N; Urbanova, M; Orn, S; Blahova, J; Svobodova, Z; Faldyna, M; Chloupek, P; Briedikova, K; Carlsson, G	Effects of antidepressants with different modes of action on early life stages of fish and amphibians	ENVIRONMENTAL POLLUTION	10.1016/j.envpol.2019.112999
2019	Kataoka, C; Sugiyama, T; Kitagawa, H; Takeshima, A; Kagami, Y; Tatsuta, H; Kashiwada, S	Temperature-dependent toxicity of acetaminophen in Japanese medaka larvae	ENVIRONMENTAL POLLUTION	10.1016/j.envpol.2019.113092
2019	de Medeiros, EF; Uehara, SA; de Freitas, TM; de Melo, NFAC; Palheta, GDA; Takata, R	Effectiveness of benzocaine as anesthetic at different water temperatures for early juvenile curimba (*Prochilodus lineatus* Valenciennes, 1836), a neotropical fish species	BOLETIM DO INSTITUTO DE PESCA	10.20950/1678-2305.2019.45.3.474
2019	Abbey-Lee, RN; Kreshchenko, A; Sala, XF; Petkova, I; Lovlie, H	Effects of monoamine manipulations on the personality and gene expression of three-spined sticklebacks	JOURNAL OF EXPERIMENTAL BIOLOGY	10.1242/jeb.211888
2019	Fraz, S; Lee, AH; Pollard, S; Srinivasan, K; Vermani, A; David, E; Wilson, JY	Paternal Exposure to Carbamazepine Impacts Zebrafish Offspring Reproduction Over Multiple Generations	ENVIRONMENTAL SCIENCE & TECHNOLOGY	10.1021/acs.est.9b03393
2019	Vera-Chang, MN; Moon, TW; Trudeau, VL	Cortisol disruption and transgenerational alteration in the expression of stress-related genes in zebrafish larvae following fluoxetine exposure	TOXICOLOGY AND APPLIED PHARMACOLOGY	10.1016/j.taap.2019.114742
2019	Thomson, JS; Al-Temeemy, AA; Isted, H; Spencer, JW; Sneddon, LU	Assessment of behaviour in groups of zebrafish (*Danio rerio*) using an intelligent software monitoring tool, the chromatic fish analyser	JOURNAL OF NEUROSCIENCE METHODS	10.1016/j.jneumeth.2019.108433
2019	Mehdi, H; Bragg, LM; Servos, MR; Craig, PM	Multiple Stressors in the Environment: The Effects of Exposure to an Antidepressant (Venlafaxine) and Increased Temperature on Zebrafish Metabolism	FRONTIERS IN PHYSIOLOGY	10.3389/fphys.2019.01431
2019	Martin, JM; Saaristo, M; Tan, H; Bertram, MG; Nagarajan-Radha, V; Dowling, DK; Wong, BBM	Field-realistic antidepressant exposure disrupts group foraging dynamics in mosquitofish	BIOLOGY LETTERS	10.1098/rsbl.2019.0615
2019	Vera-Chang, MN; Moon, TW; Trudeau, VL	Ancestral Fluoxetine Exposure Sensitizes Zebrafish to Venlafaxine-Induced Reductions in Cortisol and Spawning	ENDOCRINOLOGY	10.1210/en.2019-00281
2019	Parrish, SC; Dormio, SM; Richards, SL; McCoy, KA; McCoy, MW	Life in a contaminant milieu: PPCP mixtures generate unpredictable outcomes across trophic levels and life stages	ECOSPHERE	10.1002/ecs2.2970
2018	Yang, M; Liu, S; Hu, L; Zhan, J; Lei, PH; Wu, MH	Effects of the antidepressant, mianserin, on early development of fish embryos at low environmentally relevant concentrations	ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY	10.1016/j.ecoenv.2017.12.024
2018	Morthorst, JE; Lund, BF; Holbech, H; Bjerregaard, P	Two common mild analgesics have no effect on general endocrine mediated endpoints in zebrafish (*Danio rerio*)	COMPARATIVE BIOCHEMISTRY AND	10.1016/j.cbpc.2017.11.009

			PHYSIOLOGY C-TOXICOLOGY & PHARMACOLOGY	
2018	Zahid, H; Tsang, B; Ahmed, H; Lee, RCY; Tran, S; Gerlai, R	Diazepam fails to alter anxiety-like responses but affects motor function in a white-black test paradigm in larval zebrafish (*Danio rerio*)	PROGRESS IN NEUROPSYCHOPHARMACOLOGY & BIOLOGICAL PSYCHIATRY	10.1016/j.pnpbp.2018.01.012
2018	Pounder, KC; Mitchell, JL; Thomson, JS; Pottinger, TG; Sneddon, LU	Physiological and behavioural evaluation of common anaesthesia practices in the rainbow trout	APPLIED ANIMAL BEHAVIOUR SCIENCE	10.1016/j.applanim.2017.10.014
2018	Parrott, JL; Metcalfe, CD	Nest-defense behaviors in fathead minnows after lifecycle exposure to the antidepressant venlafaxine	ENVIRONMENTAL POLLUTION	10.1016/j.envpol.2017.11.049
2018	Gutierrez, HC; Vacca, I; Pons, AI; Norton, WHJ	Automatic quantification of juvenile zebrafish aggression	JOURNAL OF NEUROSCIENCE METHODS	10.1016/j.jneumeth.2017.12.012
2018	Sinyakova, NA; Kulikova, EA; Englevskii, NA; Kulikov, AV	Effects of Fluoxetine and Potential Antidepressant 8-Trifluoromethyl 1,2,3,4,5-Benzopentathiepin-6-Amine Hydrochloride (TC-2153) on Behavior of *Danio rerio* Fish in the Novel Tank Test and Brain Content of Biogenic Amines and Their Metabolites	BULLETIN OF EXPERIMENTAL BIOLOGY AND MEDICINE	10.1007/s10517-018-4045-6
2018	Kislyuk, S; Van den Bosch, W; Adams, E; de Witte, P; Cabooter, D	Development of a sensitive and quantitative capillary LC-UV method to study the uptake of pharmaceuticals in zebrafish brain	ANALYTICAL AND BIOANALYTICAL CHEMISTRY	10.1007/s00216-018-0955-4
2018	Nielsen, SV; Kellner, M; Henriksen, PG; Olsen, H; Hansen, SH; Baatrup, E	The psychoactive drug Escitalopram affects swimming behaviour and increases boldness in zebrafish (*Danio rerio*)	ECOTOXICOLOGY	10.1007/s10646-018-1920-x
2018	Fraz, S; Lee, AH; Wilson, JY	Gemfibrozil and carbamazepine decrease steroid production in zebrafish testes (*Danio rerio*)	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2018.02.006
2018	Choi, E; Alsop, D; Wilson, JY	The effects of chronic acetaminophen exposure on the kidney, gill and liver in rainbow trout (*Oncorhynchus mykiss*)	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2018.02.007
2018	Liao, PH; Yang, WK; Yang, CH; Lin, CH; Hwang, CC; Chen, PJ	Illicit drug ketamine induces adverse effects from behavioral alterations and oxidative stress to p53-regulated apoptosis in medaka fish under environmentally relevant exposures	ENVIRONMENTAL POLLUTION	10.1016/j.envpol.2017.11.026
2018	Mitjana, O; Bonastre, C; Tejedor, MT; Garza, L; Esteban, J; Falceto, MV	Simultaneous effect of sex and dose on efficacy of clove oil, tricaine methanesulfonate, 2-phenoxyethanol and propofol as anaesthetics in guppies, Poecilia reticulata (Peters)	AQUACULTURE RESEARCH	10.1111/are.13668
2018	Renuka, S; Poopal, RK; Ramesh, M; Clara-Bindu, F	Responses of *Labeo rohita* fingerlings to N-acetyl-p-aminophenol toxicity	ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY	10.1016/j.ecoenv.2018.03.058
2018	Bilandzija, H; Abraham, L; Ma, L; Renner, KJ; Jeffery, WR	Behavioural changes controlled by catecholaminergic systems explain recurrent loss of pigmentation in cavefish	PROCEEDINGS OF THE ROYAL SOCIETY B-BIOLOGICAL SCIENCES	10.1098/rspb.2018.0243

2018	Rozynski, M; Demska-Zakes, K; Sikora, A; Zakes, Z	Impact of inducing general anesthesia with Propiscin (etomidate) on the physiology and health of European perch (*Perca fluviatilis* L.)	FISH PHYSIOLOGY AND BIOCHEMISTRY	10.1007/s10695-018-0482-4
2018	Maulvault, AL; Santos, LHMLM; Paula, JR; Camacho, C; Pissarra, V; Fogaca, F; Barbosa, V; Alves, R; Ferreira, PP; Barcelo, D; Rodriguez-Mozaz, S; Marques, A; Diniz, M; Rosa, R	Differential behavioural responses to venlafaxine exposure route, warming and acidification in juvenile fish (*Argyrosomus regius*)	SCIENCE OF THE TOTAL ENVIRONMENT	10.1016/j.scitotenv.2018.04.015
2018	Amador, MHB; Schauer, KL; McDonald, MD	Does fluoxetine exposure affect hypoxia tolerance in the Gulf toadfish, *Opsanus beta*?	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2018.03.023
2018	Pereira, BVR; Matus, GN; Costa, MJ; Dos Santos, ACA; Silva-Zacarin, ECM; do Carmo, JB; Nunes, B	Assessment of biochemical alterations in the neotropical fish species *Phalloceros harpagos* after acute and chronic exposure to the drugs paracetamol and propranolol	ENVIRONMENTAL SCIENCE AND POLLUTION RESEARCH	10.1007/s11356-018-1699-6
2018	Bertram, MG; Ecker, TE; Wong, BBM; O'Bryan, MK; Baumgartner, JB; Martin, JM; Saaristo, M	The antidepressant fluoxetine alters mechanisms of pre- and post-copulatory sexual selection in the eastern mosquitofish (*Gambusia holbrooki*)	ENVIRONMENTAL POLLUTION	10.1016/j.envpol.2018.03.006
2018	Chen, HX; Zeng, XF; Mu, L; Hou, LP; Yang, B; Zhao, JL; Schlenk, D; Dong, W; Xie, LT; Zhang, QR	Effects of acute and chronic exposures of fluoxetine on the Chinese fish, topmouth gudgeon *Pseudorasbora parva*	ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY	10.1016/j.ecoenv.2018.04.061
2018	Rozynski, M; Hopko, M; Stawecki, K; Zakes, Z	Impact of fish size, water temperature, and MS-222 concentration on inducing general anesthesia in pikeperch (*Sander lucioperca*)	AQUACULTURE RESEARCH	10.1111/are.13738
2018	Steele, WB; Kristofco, LA; Corrales, J; Saari, GN; Haddad, SP; Gallagher, EP; Kavanagh, TJ; Kostal, J; Zimmerman, JB; Voutchkova-Kostal, A; Anastas, P; Brooks, B	Comparative behavioral toxicology with two common larval fish models: Exploring relationships among modes of action and locomotor responses	SCIENCE OF THE TOTAL ENVIRONMENT	10.1016/j.scitotenv.2018.05.402
2018	Pan, CY; Yang, M; Xu, H; Xu, BT; Jiang, LH; Wu, MH	Tissue bioconcentration and effects of fluoxetine in zebrafish (*Danio rerio*) and red crucian cap (*Carassius auratus*) after short-term and long-term exposure	CHEMOSPHERE	10.1016/j.chemosphere.2018.04.082
2018	Soltanian, S; Hoseinifar, SH; Gholamhosseini, A	Modulation of rainbow trout (*Oncorhynchus mykiss*) cutaneous mucosal immune responses following anesthesia: A comparative study on different anesthetic agents	FISH & SHELLFISH IMMUNOLOGY	10.1016/j.fsi.2018.06.032
2018	Maulvault, AL; Santos, LHMLM; Camacho, C; Anacleto, P; Barbosa, V; Alves, R; Ferreira, PP; Serra-Compte, A; Barcelo, D; Rodriguez-Mozaz, S; Rosa, R; Diniz, M; Marques, A	Antidepressants in a changing ocean: Venlafaxine uptake and elimination in juvenile fish (*Argyrosomus regius*) exposed to warming and acidification conditions	CHEMOSPHERE	10.1016/j.chemosphere.2018.06.004

2018	Meijide, FJ; Da Cuna, RH; Prieto, JP; Dorelle, LS; Babay, PA; Lo Nostro, FL	Effects of waterborne exposure to the antidepressant fluoxetine on swimming, shoaling and anxiety behaviours of the mosquitofish *Gambusia holbrooki*	ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY	10.1016/j.ecoenv.2018.07.085
2018	Oda, A; Messenger, KM; Carbajal, L; Posner, LP; Gardner, BR; Hammer, SH; Cerreta, AJ; Lewbart, GA; Bailey, KM	Pharmacokinetics and pharmacodynamic effects in koi carp (*Cyprinus carpio*) following immersion in propofol	VETERINARY ANAESTHESIA AND ANALGESIA	10.1016/j.vaa.2018.02.005
2018	Steele, WB; Mole, RA; Brooks, BW	Experimental Protocol for Examining Behavioral Response Profiles in Larval Fish: Application to the Neuro-stimulant Caffeine	JOVE-JOURNAL OF VISUALIZED EXPERIMENTS	10.3791/57938
2018	Romaneli, RD; Boaratti, AZ; Rodrigues, AT; Queiroz, DMD; Khan, KU; Nascimento, TMT; Fernandes, JBK; Mansano, CFM	Efficacy of Benzocaine, Eugenol, and Menthol as Anesthetics for Freshwater Angelfish	JOURNAL OF AQUATIC ANIMAL HEALTH	10.1002/aah.10030
2018	Matus, GN; Pereira, BVR; Silva-Zacarin, ECM; Costa, MJ; dos Santos, ACA; Nunes, B	Behavior and histopathology as biomarkers for evaluation of the effects of paracetamol and propranolol in the neotropical fish species *Phalloceros harpagos*	ENVIRONMENTAL SCIENCE AND POLLUTION RESEARCH	10.1007/s11356-018-2839-8
2018	Chin, JSR; Gassant, CE; Amaral, PM; Lloyd, E; Stahl, BA; Jaggard, JB; Keene, AC; Duboue, ER	Convergence on reduced stress behavior in the Mexican blind cavefish	DEVELOPMENTAL BIOLOGY	10.1016/j.ydbio.2018.05.009
2018	Michelotti, P; Quadros, VA; Pereira, ME; Rosemberg, DB	Ketamine modulates aggressive behavior in adult zebrafish	NEUROSCIENCE LETTERS	10.1016/j.neulet.2018.08.009
2018	Zhai, SW; Shepherd, BS; Yang, S; Zhao, HH; Jiang, M; Binkowski, FP; Deng, DF	Efficacy of Tricaine Methanesulfonate (MS-222) as an Anesthetic Agent for Short-term Anesthesia in Juvenile Yellow Perch (*Perca flavescens*)	ISRAELI JOURNAL OF AQUACULTURE-BAMIDGEH	10.46989/001c.20923
2018	Kucuk, S	Effects of tricaine on blue tilapia at different salinities and concentrations	SCIENTIFIC PAPERS-SERIES D-ANIMAL SCIENCE	
2018	Trushenski, JT; Johnson, JA; Bowker, JD	Efficacy and Hematological Responses of Walleyes to Chemosedation and Electrosedation	NORTH AMERICAN JOURNAL OF AQUACULTURE	10.1002/naaq.10040
2018	Ros, N; Lomba, L; Ribate, MP; Zuriaga, E; Garcia, CB; Giner, B	Acute lethal and sublethal effects of diltiazem and doxepin for four aquatic environmental bioindicators covering the trophic chain	AIMS ENVIRONMENTAL SCIENCE	10.3934/environsci.2018.4.229
2018	Santos, ND; Oliveira, R; Lisboa, CA; Pinto, JME; Sousa-Moura, D; Camargo, NS; Perillo, V; Oliveira, M; Grisolia, CK; Domingues, I	Chronic effects of carbamazepine on zebrafish: Behavioral, reproductive and biochemical endpoints	ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY	10.1016/j.ecoenv.2018.08.015
2018	Zellar, AK; Olea-Popelka, FJ; Campbell, TW	A comparison of alfaxalone and tricaine methanesulphonate (ms-222) in two fish species	JOURNAL OF EXOTIC PET MEDICINE	10.1053/j.jepm.2018.06.002

2018	Yan, SH; Wang, M; Liang, XF; Martyniuk, CJ; Zha, JM; Wang, ZJ	Environmentally relevant concentrations of carbamazepine induce liver histopathological changes and a gender-specific response in hepatic proteome of Chinese rare minnows (*Gobiocypris rarus*)	ENVIRONMENTAL POLLUTION	10.1016/j.envpol.2018.09.009
2018	Vera-Chang, MN; St-Jacques, AD; Gagne, R; Martyniuk, CJ; Yauk, CL; Moon, TW; Trudeau, VL	Transgenerational hypocortisolism and behavioral disruption are induced by the antidepressant fluoxetine in male zebrafish *Danio rerio*	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA	10.1073/pnas.1811695115
2018	Kim, EJ; Nam, YK	Anesthetic protocol for microinjection-related handling of Siberian sturgeon (*Acipenser baerii*; Acipenseriformes) prolarvae	PLOS ONE	10.1371/journal.pone.0209928
2018	Hodkovicova, N; Urbanova, M; Sehonova, P; Chloupek, P	The effect of antidepressants in surface water on *Danio rerio* organism	PROCEEDINGS OF 25TH INTERNATIONAL PHD STUDENTS CONFERENCE (MENDELNET 2018)	
2018	Kellner, M; Porseryd, T; Porsch-Hallstrom, I; Borg, B; Roufidou, C; Olsen, KH	Developmental exposure to the SSRI citalopram causes longlasting behavioural effects in the three-spined stickleback (*Gasterosteus aculeatus*)	ECOTOXICOLOGY	10.1007/s10646-017-1866-4
2017	Dong, C; Pan, L; He, D; Xie, JJ; Tang, HY; Yang, Z; Wang, XM; Yang, SR	The Efficacy of MS-222 as Anesthetic Agent in Largemouth Bronze Gudgeon Coreius guichenoti	NORTH AMERICAN JOURNAL OF AQUACULTURE	10.1080/15222055.2016.1245228
2017	Cunha, V; Burkhardt-Medicke, K; Wellner, P; Santos, MM; Moradas-Ferreira, P; Luckenbach, T; Ferreira, M	Effects of pharmaceuticals and personal care products (ppcps) on multixenobiotic resistance (MXR) related efflux transporter activity in zebrafish (*Danio rerio*) embryos	ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY	10.1016/j.ecoenv.2016.10.022
2017	Melvin, SD	Effect of antidepressants on circadian rhythms in fish: Insights and implications regarding the design of behavioural toxicity tests.	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2016.11.007
2017	Huang, SSY; Benskin, JP; Veldhoen, N; Chandramouli, B; Butler, H; Helbing, CC; Cosgrove, JR	A multi-omic approach to elucidate low-dose effects of xenobiotics in zebrafish (*Danio rerio*) larvae	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2016.11.016
2017	Mottaz, H; Schonenberger, R; Fischer, S; Eggen, RIL; Schirmer, K; Groh, KJ	Dose-dependent effects of morphine on lipopolysaccharide (LPS)-induced inflammation, and involvement of multixenobiotic resistance (MXR) transporters in LPS efflux in teleost fish	ENVIRONMENTAL POLLUTION	10.1016/j.envpol.2016.11.046
2017	Wynd, BM; Watson, CJ; Patil, K; Sanders, GE; Kwon, RY	A Dynamic Anesthesia System for Long-Term Imaging in Adult Zebrafish	ZEBRAFISH	10.1089/zeb.2016.1289

2017	Wang, LW; Huttner, IG; Santiago, CF; Kesteven, SH; Yu, ZY; Feneley, MP; Fatkin, D	Standardized echocardiographic assessment of cardiac function in normal adult zebrafish and heart disease models	DISEASE MODELS & MECHANISMS	10.1242/dmm.026989
2017	Saaristo, M; McLennan, A; Johnstone, CP; Clarke, BO; Wong, BBM	Impacts of the antidepressant fluoxetine on the anti-predator behaviours of wild guppies (*Poecilia reticulata*)	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2016.12.007
2017	Schroeder, PG; Sneddon, LU	Exploring the efficacy of immersion analgesics in zebrafish using an integrative approach	APPLIED ANIMAL BEHAVIOUR SCIENCE	10.1016/j.applanim.2016.12.003
2017	Stancova, V; Plhalova, L; Blahova, J; Zivna, D; Bartoskova, M; Siroka, Z; Marsalek, P; Svobodova, Z	Effects of the pharmaceutical contaminants ibuprofen, diclofenac, and carbamazepine alone, and in combination, on oxidative stress parameters in early life stages of tench (*Tinca tinca*)	VETERINARNI MEDICINA	10.17221/125/2016-VETMED
2017	Witeska, M; Teodorczuk, B; Lugowska, K	Hematological effects of etomidate and tricaine in common carp	TURKISH JOURNAL OF VETERINARY & ANIMAL SCIENCES	10.3906/vet-1603-30
2017	Ziarrusta, H; Mijangos, L; Izagirre, U; Plassmann, MM; Benskin, JP; Anakabe, E; Olivares, M; Zuloaga, O	Bioconcentration and Biotransformation of Amitriptyline in Gilt-Head Bream	ENVIRONMENTAL SCIENCE & TECHNOLOGY	10.1021/acs.est.6b05831
2017	Martin, JM; Saaristo, M; Bertram, MG; Lewis, PJ; Coggan, TL; Clarke, BO; Wong, BBM	The psychoactive pollutant fluoxetine compromises antipredator behaviour in fish	ENVIRONMENTAL POLLUTION	10.1016/j.envpol.2016.10.010
2017	Rucinque, DS; Polo, G; Borbon, J; Mantilla, JFG	Anesthetic use of eugenol and benzocaine in juveniles of red tilapia (*Oreochromis* sp.)	REVISTA COLOMBIANA DE CIENCIAS PECUARIAS	10.17533/udea.rccp.v30n1a07
2017	Tran, S; Fulcher, N; Nowicki, M; Desai, P; Tsang, B; Facciol, A; Chow, H; Gerlai, R	Time-dependent interacting effects of caffeine, diazepam, and ethanol on zebrafish behaviour	PROGRESS IN NEUROPSYCHOPHARMACOLOGY & BIOLOGICAL PSYCHIATRY	10.1016/j.pnpbp.2016.12.004
2017	McCallum, ES; Bose, APH; Warriner, TR; Balshine, S	An evaluation of behavioural endpoints: The pharmaceutical pollutant fluoxetine decreases aggression across multiple contexts in round goby (*Neogobius melanostomus*)	CHEMOSPHERE	10.1016/j.chemosphere.2017.02.059
2017	Bostrom, ML; Ugge, G; Jonsson, JA; Berglund, O	Bioaccumulation and trophodynamics of the antidepressants sertraline and fluoxetine in laboratory-constructed, 3-level aquatic food chains	ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY	10.1002/etc.3637
2017	HedayatiRad, M; Nematollahi, MA; Forsatkar, MN; Brown, C	Prozac impacts lateralization of aggression in male Siamese fighting fish	ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY	10.1016/j.ecoenv.2017.02.027
2017	Lopez-Luna, J; Al-Jubouri, Q; Al-Nuaimy, W; Sneddon, LU	Reduction in activity by noxious chemical stimulation is ameliorated by immersion in analgesic drugs in zebrafish	JOURNAL OF EXPERIMENTAL BIOLOGY	10.1242/jeb.146969

2017	Zhao, JL; Furlong, ET; Schoenfuss, HL; Kolpin, DW; Bird, KL; Feifarek, DJ; Schwab, EA; Ying, GG	Uptake and Disposition of Select Pharmaceuticals by Bluegill Exposed at Constant Concentrations in a Flow-Through Aquatic Exposure System	ENVIRONMENTAL SCIENCE & TECHNOLOGY	10.1021/acs.est.7b00604
2017	Balko, JA; Wilson, SK; Lewbart, GA; Gaines, BR; Posner, LP	Propofol as an immersion anesthetic and in a minimum anesthetic concentration (mac) reduction model in goldfish (*Carassius auratus*)	JOURNAL OF ZOO AND WILDLIFE MEDICINE	10.1638/2016-0079.1
2017	Huang, SY; Xu, JQ; Wu, JY; Hong, HJ; Chen, GS; Jiang, RF; Zhu, F; Liu, Y; Ouyang, GF	Rapid detection of five anesthetics in tilapias by *in vivo* solid phase microextraction coupling with gas chromatography-mass spectrometry	TALANTA	10.1016/j.talanta.2017.03.045
2017	Carty, DR; Hala, D; Huggett, DB	The Effects of Sertraline on Fathead Minnow (*Pimephales promelas*) Growth and Steroidogenesis	BULLETIN OF ENVIRONMENTAL CONTAMINATION AND TOXICOLOGY	10.1007/s00128-017-2079-5
2017	de Boissel, PGJ; Gonzalez, P; Bulete, A; Daffe, G; Clerandeau, C; Vulliet, E; Cachot, J	An innovative and integrative assay for toxicity testing using individual fish embryos. Application to oxazepam	CHEMOSPHERE	10.1016/j.chemosphere.2017.04.067
2017	Aguirre-Martinez, GV; Reinardy, HC; Martin-Diaz, ML; Henry, TB	Response of gene expression in zebrafish exposed to pharmaceutical mixtures: Implications for environmental risk	ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY	10.1016/j.ecoenv.2017.04.038
2017	Kacprzak, V; Patel, NA; Riley, E; Yu, LL; Yeh, JRJ; Zhdanova, IV	Dopaminergic control of anxiety in young and aged zebrafish	PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR	10.1016/j.pbb.2017.01.005
2017	Xia, L; Zheng, L; Zhou, JL	Effects of ibuprofen, diclofenac and paracetamol on hatch and motor behavior in developing zebrafish (*Danio rerio*)	CHEMOSPHERE	10.1016/j.chemosphere.2017.05.054
2017	Skar, MW; Haugland, GT; Powell, MD; Wergeland, HI; Samuelsen, OB	Development of anaesthetic protocols for lumpfish (*Cyclopterus lumpus* L.): Effect of anaesthetic concentrations, sea water temperature and body weight	PLOS ONE	10.1371/journal.pone.0179344
2017	Kayhan, FE; Duman, BS; Kaymak, G; Gunduz, MK; Tartar, S; Esmer, HE; Akbulut, C; Cevik, M; Ertug, NDY	The sublethal disrupting effects of fluoxetine-hcl (flx) on catalase (cat) activity and malondialdehyde (mda) levels in zebrafish, *Danio rerio*	FRESENIUS ENVIRONMENTAL BULLETIN	
2017	Theodoridi, A; Tsalafouta, A; Pavlidis, M	Acute Exposure to Fluoxetine Alters Aggressive Behavior of Zebrafish and Expression of Genes Involved in Serotonergic System Regulation	FRONTIERS IN NEUROSCIENCE	10.3389/fnins.2017.00223
2017	Guiloski, IC; Ribas, JLC; Piancini, LDS; Dagostim, AC; Cirio, SM; Favaro, LF; Boschen, SL; Cestari, MM; da Cunha, C; de Assis, HCS	Paracetamol causes endocrine disruption and hepatotoxicity in male fish *Rhamdia quelen* after subchronic exposure	ENVIRONMENTAL TOXICOLOGY AND PHARMACOLOGY	10.1016/j.etap.2017.05.005
2017	Matsche, MA	Efficacy and Physiological Response to Chemical Anesthesia in Wild Hickory Shad during Spawning Season	MARINE AND COASTAL FISHERIES	10.1080/19425120.2017.1321593

2017	Sehonova, P; Plhalova, L; Blahova, J; Doubkova, V; Marsalek, P; Prokes, M; Tichy, F; Skladana, M; Fiorino, E; Mikula, P; Vecerek, V; Faggio, C; Svobodova, Z	Effects of selected tricyclic antidepressants on early-life stages of common carp (*Cyprinus carpio*)	CHEMOSPHERE	10.1016/j.chemosphere.2017.07.092
2017	Parrott, JL; Metcalfe, CD	Assessing the effects of the antidepressant venlafaxine to fathead minnows exposed to environmentally relevant concentrations over a full life cycle	ENVIRONMENTAL POLLUTION	10.1016/j.envpol.2017.06.009
2017	Nahon, S; Seite, S; Kolasinski, J; Aguirre, P; Geurden, I	Effects of euthanasia methods on stable carbon (C-13 value) and nitrogen (N-15 value) isotopic compositions of fry and juvenile rainbow trout *Oncorhynchus mykiss*	RAPID COMMUNICATIONS IN MASS SPECTROMETRY	10.1002/rcm.7958
2017	Weichert, FG; Floeter, C; Artmann, ASM; Karnmann, U	Assessing the ecotoxicity of potentially neurotoxic substances - Evaluation of a behavioural parameter in the embryogenesis of *Danio rerio*	CHEMOSPHERE	10.1016/j.chemosphere.2017.07.136
2017	Mishra, P; Gong, ZY; Kelly, BC	Assessing biological effects of fluoxetine in developing zebrafish embryos using gas chromatography-mass spectrometry based metabolomics	CHEMOSPHERE	10.1016/j.chemosphere.2017.08.149
2017	Kalichak, F; Idalencio, R; da Rosa, JGS; Barcellos, HHD; Fagundes, M; Piato, A; Barcellos, LJG	Psychotropic in the environment: risperidone residues affect the behavior of fish larvae	SCIENTIFIC REPORTS	10.1038/s41598-017-14575-7
2017	Mennigen, JA; Zamora, JM; Chang, JP; Trudeau, VL	Endocrine disrupting effects of waterborne fluoxetine exposure on the reproductive axis of female goldfish, *Carassius auratus*	COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY C-TOXICOLOGY & PHARMACOLOGY	10.1016/j.cbpc.2017.08.003
2017	Tanoue, R; Margiotta-Casaluci, L; Huerta, B; Runnalls, TJ; Nomiyama, K; Kunisue, T; Tanabe, S; Sumpter, JP	Uptake and Metabolism of Human Pharmaceuticals by Fish: A Case Study with the Opioid Analgesic Tramadol	ENVIRONMENTAL SCIENCE & TECHNOLOGY	10.1021/acs.est.7b03441
2017	Cassar, S; Breidenbach, L; Olson, A; Huang, X; Britton, H; Woody, C; Sancheti, P; Stolarik, D; Wicke, K; Hempel, K; LeRoy, B	Measuring drug absorption improves interpretation of behavioral responses in a larval zebrafish locomotor assay for predicting seizure liability	JOURNAL OF PHARMACOLOGICAL AND TOXICOLOGICAL METHODS	10.1016/j.vascn.2017.07.002
2017	Wu, MH; Liu, S; Hu, L; Qu, HD; Pan, CY; Lei, PH; Shen, YJ; Yang, M	Global transcriptomic analysis of zebrafish in response to embryonic exposure to three antidepressants, amitriptyline, fluoxetine and mianserin	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2017.09.027
2017	Stringhetta, GR; Barbas, LAL; Maltez, LC; Sampaio, LA; Monserrat, JM; Garcia, LO	Oxidative stress responses of juvenile tambaqui *Colossoma macropomum* after short-term anesthesia with benzocaine and MS-222	ANAIS DA ACADEMIA BRASILEIRA DE CIENCIAS	10.1590/0001-3765201720160823

2017	Brodin, T; Nordling, J; Lagesson, A; Klaminder, J; Hellstrom, G; Christensen, B; Fick, J	Environmental relevant levels of a benzodiazepine (oxazepam) alters important behavioral traits in a common planktivorous fish, (*Rutilus rutilus*)	JOURNAL OF TOXICOLOGY AND ENVIRONMENTAL HEALTH-PART A-CURRENT ISSUES	10.1080/15287394.2017.1352214
2017	Porseryd, T; Kellner, M; Caspillo, NR; Volkova, K; Elabbas, L; Ullah, S; Olsen, H; Dinnetz, P; Hallstrom, IP	Combinatory effects of low concentrations of 17 alpha-etinylestradiol and citalopram on non-reproductive behavior in adult zebrafish (*Danio rerio*)	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2017.10.001
2017	Bolasina, SN; de Azevedo, A; Petry, AC	Comparative efficacy of benzocaine, tricaine methanesulfonate and eugenol as anesthetic agents in the guppy *Poecilia vivipara*	AQUACULTURE REPORTS	10.1016/j.aqrep.2017.04.002
2016	Giacomini, ACVV; Abreu, MS; Giacomini, LV; Siebel, AM; Zimerman, FF; Rambo, CL; Mocelin, R; Bonan, CD; Piato, AL; Barcellos, LJG	Fluoxetine and diazepam acutely modulate stress induced-behavior	BEHAVIOURAL BRAIN RESEARCH	10.1016/j.bbr.2015.09.027
2016	Abreu, MS; Giacomini, ACV; Gusso, D; Rosa, JGS; Koakoski, G; Kalichak, F; Idalencio, R; Oliveira, TA; Barcellos, HHA; Bonan, CD; Barcellos, LJG	Acute exposure to waterborne psychoactive drugs attract zebrafish	COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY C-TOXICOLOGY & PHARMACOLOGY	10.1016/j.cbpc.2015.08.009
2016	Berlinsky, DL; Watson, MT; DiMaggio, MA; Breton, TS	The Use of Tricaine Methanesulfonate, Clove Oil, Metomidate, and 2-Phenoxyethanol for Anesthesia Induction in Alewives	NORTH AMERICAN JOURNAL OF AQUACULTURE	10.1080/15222055.2015.1105891
2016	Dzieweczynski, TL; Kane, JL; Campbell, BA; Lavin, LE	Fluoxetine exposure impacts boldness in female Siamese fighting fish, *Betta splendens*	ECOTOXICOLOGY	10.1007/s10646-015-1568-8
2016	Ding, JN; Lu, GH; Li, Y	Interactive effects of selected pharmaceutical mixtures on bioaccumulation and biochemical status in crucian carp (*Carassius auratus*)	CHEMOSPHERE	10.1016/j.chemosphere.2016.01.017
2016	Li, L; Bonneton, F; Tohme, M; Bernard, L; Chen, XY; Laudet, V	*In Vivo* Screening Using Transgenic Zebrafish Embryos Reveals New Effects of HDAC Inhibitors Trichostatin A and Valproic Acid on Organogenesis	PLOS ONE	10.1371/journal.pone.0149497
2016	Nallani, GC; Edziyie, RE; Paulos, PM; Venables, BJ; Constantine, LA; Huggett, DB	Bioconcentration of two basic pharmaceuticals, verapamil and clozapine, in fish	ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY	10.1002/etc.3244
2016	Bugman, AM; Langer, PT; Hadzima, E; Rivas, AE; Mitchell, MA	Evaluation of the anesthetic efficacy of alfaxalone in oscar fish (*Astronotus ocellatus*)	AMERICAN JOURNAL OF VETERINARY RESEARCH	10.2460/ajvr.77.3.239
2016	Dzieweczynski, TL; Campbell, BA; Kane, JL	Dose-dependent fluoxetine effects on boldness in male Siamese fighting fish	JOURNAL OF EXPERIMENTAL BIOLOGY	10.1242/jeb.132761

2016	Heynen, M; Fick, J; Jonsson, M; Klaminder, J; Brodin, T	Effect of bioconcentration and trophic transfer on realized exposure to oxazepam in 2 predators, the dragonfly larvae (*Aeshna grandis*) and the eurasian perch (*Perca fluviatilis*)	ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY	10.1002/etc.3368
2016	Schoenfuss, HL; Furlong, ET; Phillips, PJ; Scott, TM; Kolpin, DW; Cetkovic-Cvrlje, M; Lesteberg, KE; Rearick, DC	Complex mixtures, complex responses: assessing pharmaceutical mixtures using field and laboratory approaches	ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY	10.1002/etc.3147
2016	Watanabe, H; Tamura, I; Abe, R; Takanobu, H; Nakamura, A; Suzuki, T; Hirose, A; Nishimura, T; Tatarazako, N	Chronic toxicity of an environmentally relevant mixture of pharmaceuticals to three aquatic organisms (alga, daphnid, and fish)	ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY	10.1002/etc.3285
2016	Santos, LC; -Oliveira, JR; Oliveira, JJ; Silva, PF; Luchiari, AC	Irish coffee: Effects of alcohol and caffeine on object discrimination in zebrafish	PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR	10.1016/j.pbb.2016.01.013
2016	Ansai, S; Hosokawa, H; Maegawa, S; Kinoshita, M	Chronic fluoxetine treatment induces anxiolytic responses and altered social behaviors in medaka, *Oryzias latipes*	BEHAVIOURAL BRAIN RESEARCH	10.1016/j.bbr.2016.01.050
2016	Kellner, M; Porseryd, T; Hallgren, S; Porsch-Hallstrom, I; Hansen, SH; Olsen, KH	Waterborne citalopram has anxiolytic effects and increases locomotor activity in the three-spine stickleback (*Gasterosteus aculeatus*)	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2015.12.026
2016	Abreu, MS; Giacomini, ACVV; Kalueff, AV; Barcellos, LJG	The smell of "anxiety": Behavioral modulation by experimental anosmia in zebrafish	PHYSIOLOGY & BEHAVIOR	10.1016/j.physbeh.2016.01.030
2016	Mazelet, L; Parker, MO; Li, M; Arner, A; Ashworth, R	Role of Active Contraction and Tropomodulins in Regulating Actin Filament Length and Sarcomere Structure in Developing Zebrafish Skeletal Muscle	FRONTIERS IN PHYSIOLOGY	10.3389/fphys.2016.00091
2016	Overturf, CL; Overturf, MD; Huggett, DB	Bioconcentration and endocrine disruption effects of diazepam in channel catfish, *Ictalurus punctatus*	COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY C-TOXICOLOGY & PHARMACOLOGY	10.1016/j.cbpc.2016.02.001
2016	Chiffre, A; Clerandeau, C; Dwoinikoff, C; Le Bihanic, F; Budzinski, H; Geret, F; Cachot, J	Psychotropic drugs in mixture alter swimming behaviour of Japanese medaka (*Oryzias latipes*) larvae above environmental concentrations	ENVIRONMENTAL SCIENCE AND POLLUTION RESEARCH	10.1007/s11356-014-3477-4
2016	Lorenzi, V; Choe, R; Schlenk, D	Effects of environmental exposure to diazepam on the reproductive behavior of fathead minnow, *Pimephales promelas*	ENVIRONMENTAL TOXICOLOGY	10.1002/tox.22069
2016	Rahbar, S; Pan, W; Jonz, MG	Purinergic and Cholinergic Drugs Mediate Hyperventilation in Zebrafish: Evidence from a Novel Chemical Screen	PLOS ONE	10.1371/journal.pone.0154261
2016	Valdes, ME; Huerta, B; Wunderlin, DA; Bistoni, MA; Barcelo, D; Rodriguez-Mozaz, S	Bioaccumulation and bioconcentration of carbamazepine and other pharmaceuticals in fish under field and controlled laboratory experiments. Evidences of carbamazepine metabolization by fish	SCIENCE OF THE TOTAL ENVIRONMENT	10.1016/j.scitotenv.2016.03.045
2016	Abreu, MS; Giacomini, ACVV; Koakoski, G; Piato, AL; Barcellos, LJG	Evaluating "anxiety" and social behavior in jundia (*Rhamdia quelen*)	PHYSIOLOGY & BEHAVIOR	10.1016/j.physbeh.2016.04.003

2016	Qin, G; Zhang, YH; Wang, X; Lin, Q	Effects of Anesthetic Disposal on the Physiological and Behavioral Responses of the Lined Seahorses, Hippocampus erectus	JOURNAL OF THE WORLD AQUACULTURE SOCIETY	10.1111/jwas.12282
2016	Giacomini, ACVV; Abreu, MS; Zanandrea, R; Saibt, N; Friedrich, MT; Koakoski, G; Gusso, D; Piato, AL; Barcellos, LJG	Environmental and Pharmacological Manipulations Blunt the Stress Response of Zebrafish in a Similar Manner	SCIENTIFIC REPORTS	10.1038/srep28986
2016	Johnson, JL; Trushenski, JT; Bowker, JD	Induction, Recovery, and Hematological Responses of Pallid Sturgeon to Chemical and Electrical Sedation	NORTH AMERICAN JOURNAL OF FISHERIES MANAGEMENT	10.1080/02755947.2016.1141121
2016	Sweet, LE; Bisesi, JH; Lei, ENY; Lam, MHW; Klaine, SJ	The effects of bupropion on hybrid striped bass brain chemistry and predatory behavior	ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY	10.1002/etc.3350
2016	Qiang, LY; Cheng, JP; Yi, J; Rotchell, JM; Zhu, XT; Zhou, JL	Environmental concentration of carbamazepine accelerates fish embryonic development and disturbs larvae behavior	ECOTOXICOLOGY	10.1007/s10646-016-1694-y
2016	Hedgespeth, ML; Nilsson, PA; Berglund, O	Assessing Potential Vulnerability and Response of Fish to Simulated Avian Predation after Exposure to Psychotropic Pharmaceuticals	TOXICS	10.3390/toxics4020009
2016	Felix, LM; Serafim, C; Valentim, AM; Antunes, LM; Campos, S; Matos, M; Coimbra, AM	Embryonic Stage-Dependent Teratogenicity of Ketamine in Zebrafish (*Danio rerio*)	CHEMICAL RESEARCH IN TOXICOLOGY	10.1021/acs.chemrestox.6b00122
2016	Zivna, D; Blahova, J; Siroka, Z; Plhalova, L; Marsalek, P; Doubkova, V; Zelinska, G; Vecerek, V; Tichy, F; Sehonova, P; Svobodova, Z	The Effects of Salicylic Acid on Juvenile Zebrafish *Danio rerio* Under Flow-Through Conditions	BULLETIN OF ENVIRONMENTAL CONTAMINATION AND TOXICOLOGY	10.1007/s00128-016-1877-5
2016	Cunha, V; Rodrigues, P; Santos, MM; Moradas-Ferreira, P; Ferreira, M	*Danio rerio* embryos on Prozac - Effects on the detoxification mechanism and embryo development	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2016.08.003
2016	Singer, ML; Oreschak, K; Rhinehart, Z; Robison, BD	Anxiolytic effects of fluoxetine and nicotine exposure on exploratory behavior in zebrafish	PEERJ	10.7717/peerj.2352
2016	Bain, PA; Basheer, VS; Gregg, A; Jena, JK; Kumar, A	*In vitro* nuclear receptor activity and *in vivo* gene expression analysis in Murray-Darling rainbow fish (*Melanotaenia fluviatilis*) after short-term exposure to fluoxetine	COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY C-TOXICOLOGY & PHARMACOLOGY	10.1016/j.cbpc.2016.05.007
2016	Brox, S; Seiwert, B; Kuster, E; Reemtsma, T	Toxicokinetics of Polar Chemicals in Zebrafish Embryo (*Danio rerio*): Influence of Physicochemical Properties and of Biological Processes	ENVIRONMENTAL SCIENCE & TECHNOLOGY	10.1021/acs.est.6b04325
2016	Heynen, M; Brodin, T; Klaminder, J; Jonsson, M; Fick, J	Tissue-specific uptake of the benzodiazepine oxazepam in adult Eurasian perch (*Perca fluviatilis*)	ENVIRONMENTAL CHEMISTRY	10.1071/EN16027

2016	Huerta, B; Margiotta-Casaluci, L; Rodriguez-Mozaz, S; Scholze, M; Winter, MJ; Barcelo, D; Sumpter, JP	Anti-anxiety drugs and fish behavior: establishing the link between internal concentrations of oxazepam and behavioral effects	ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY	10.1002/etc.3448
2016	Kazemi, MB	A comparative study on the effects of clove oil, 2-phenoxy ethanol, and MS-222 upon some enzymatic and hormonal activities in juvenile Caspian brown trout (*Salmo trutta caspius*)	INTERNATIONAL JOURNAL OF ADVANCED BIOTECHNOLOGY AND RESEARCH	
2016	Barcellos, HHD; Kalichak, F; da Rosa, JGS; Oliveira, TA; Koakoski, G; Idalencio, R; de Abreu, MS; Giacomini, ACV; Fagundes, M; Variani, C; Rossini, M; Piato, AL; Barcellos, LJG	Waterborne aripiprazole blunts the stress response in zebrafish	SCIENTIFIC REPORTS	10.1038/srep37612
2016	Kucuk, S; Coban, D	Effects of Tricaine as an Anaesthetics on Goldfish, *Carassius auratus* (Linnaeus 1758) at Different Salinities and Concentrations	TURKISH JOURNAL OF FISHERIES AND AQUATIC SCIENCES	10.4194/1303-2712-v16_3_13
2016	Rodrigues, RB; Melo, IWD; Rocha, JDM; da Silva, TC; Bridi, VRC; Signor, A; Bittencourt, F; Boscolo, WR	Induction time of anesthesia and recovery of benzocaine to patinga (*Piaractus mesopotamicus* x *Piaractus brachypomus*)	BRAZILIAN JOURNAL OF HYGIENE AND ANIMAL SANITY	10.5935/1981-2965.20160030
2016	Heynen, M; Backstrom, T; Fick, J; Jonsson, M; Klaminder, J; Brodin, T	Home alone-The effects of isolation on uptake of a pharmaceutical contaminant in a social fish	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2016.09.004
2016	Kirla, KT; Groh, KJ; Steuer, AE; Poetzsch, M; Banote, RK; Stadnicka-Michalak, J; Eggen, RIL; Schirmer, K; Kraemer, T	From the Cover: Zebrafish Larvae Are Insensitive to Stimulation by Cocaine: Importance of Exposure Route and Toxicokinetics	TOXICOLOGICAL SCIENCES	10.1093/toxsci/kfw156
2016	Hellstrom, G; Klaminder, J; Finn, F; Persson, L; Alanara, A; Jonsson, M; Fick, J; Brodin, T	Gabaergic anxiolytic drug in water increases migration behaviour in salmon	NATURE COMMUNICATIONS	10.1038/ncomms13460
2016	Adel, M; Sadegh, AB; Yeganeh, S; Movafagh, AN; Saoud, IP	Anesthetic Efficacy of Clove Oil, Propofol, 2-Phenoxyethanol, and Ketamine Hydrochloride on Persian Sturgeon, *Acipenser persicus*, Juveniles	JOURNAL OF THE WORLD AQUACULTURE SOCIETY	10.1111/jwas.12286
2016	Kalichak, F; Idalencio, R; Rosa, JGS; de Oliveira, TA; Koakoski, G; Gusso, D; de Abreu, MS; Giacomini, ACV; Barcellos, HHA; Fagundes, M; Piato, AL; Barcellos, LJG	Waterborne psychoactive drugs impair the initial development of Zebrafish	ENVIRONMENTAL TOXICOLOGY AND PHARMACOLOGY	10.1016/j.etap.2015.11.014
2016	Klaminder, J; Hellstrom, G; Fahlman, J; Jonsson, M; Fick, J; Lagesson, A; Bergman, E; Brodin, T	Drug-Induced Behavioral Changes: Using Laboratory Observations to Predict Field Observations	FRONTIERS IN ENVIRONMENTAL SCIENCE	10.3389/fenvs.2016.00081

2015	Nunes, B; Campos, JC; Gomes, R; Braga, MR; Ramos, AS; Antunes, SC; Correia, AT	Ecotoxicological effects of salicylic acid in the freshwater fish *Salmo trutta fario*: antioxidant mechanisms and histological alterations	ENVIRONMENTAL SCIENCE AND POLLUTION RESEARCH	10.1007/s11356-014-3337-2
2015	Curtright, A; Rosser, M; Goh, S; Keown, B; Wagner, E; Sharifi, J; Raible, DW; Dhaka, A	Modeling Nociception in Zebrafish: A Way Forward for Unbiased Analgesic Discovery	PLOS ONE	10.1371/journal.pone.0116766
2015	Kellner, M; Porseryd, T; Porsch-Hallstrom, I; Hansen, SH; Olsen, KH	Environmentally relevant concentrations of citalopram partially inhibit feeding in the three-spine stickleback (*Gasterosteus aculeatus*)	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2014.11.003
2015	Bowker, JD; Trushenski, JT; Glover, DC; Carty, DG; Wandelear, N	Sedative options for fish research: a brief review with new data on sedation of warm-, cool-, and coldwater fishes and recommendations for the drug approval process	REVIEWS IN FISH BIOLOGY AND FISHERIES	10.1007/s11160-014-9374-6
2015	Backstrom, T; Heynen, M; Brannas, E; Nilsson, J; Magnhagen, C	The effect of anesthetics on carotenoid pigmentation and behavior in Arctic char (*Salvelinus alpinus*)	JOURNAL OF VETERINARY BEHAVIOR-CLINICAL APPLICATIONS AND RESEARCH	10.1016/j.jveb.2014.11.007
2015	Ladu, F; Mwaffo, V; Li, J; Macri, S; Porfiri, M	Acute caffeine administration affects zebrafish response to a robotic stimulus	BEHAVIOURAL BRAIN RESEARCH	10.1016/j.bbr.2015.04.020
2015	Xie, ZX; Lu, GH; Li, S; Nie, Y; Ma, BN; Liu, JC	Behavioral and biochemical responses in freshwater fish *Carassius auratus* exposed to sertraline	CHEMOSPHERE	10.1016/j.chemosphere.2015.04.031
2015	Chen, FJ; Chen, SJ; Liu, SS; Zhang, CZ; Peng, G	Effects of lorazepam and WAY-200070 in larval zebrafish light/dark choice	NEUROPHARMACOLOGY	10.1016/j.neuropharm.2015.03.022
2015	Rodrigues, S; Correia, AT; Antunes, SC; Nunes, B	Alterations in gills of *Lepomis gibbosus*, after acute exposure to several xenobiotics (pesticide, detergent and pharmaceuticals): morphometric and biochemical evaluation	DRUG AND CHEMICAL TOXICOLOGY	10.3109/01480545.2014.918999
2015	Liao, PH; Hwang, CC; Chen, TH; Chen, PJ	Developmental exposures to waterborne abused drugs alter physiological function and larval locomotion in early life stages of medaka fish	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2015.05.010
2015	Zivna, D; Sehonova, P; Plhalova, L; Marsalek, P; Blahova, J; Prokes, M; Divisova, L; Stancova, V; Dobsikova, R; Tichy, F; Siroka, Z; Svobodova, Z	Effect of salicylic acid on early life stages of common carp (*Cyprinus carpio*)	ENVIRONMENTAL TOXICOLOGY AND PHARMACOLOGY	10.1016/j.etap.2015.06.018
2015	Schaefer, IC; Siebel, AM; Piato, AL; Bonan, CD; Vianna, MR; Lara, DR	The side-by-side exploratory test: a simple automated protocol for the evaluation of adult zebrafish behavior simultaneously with social interaction	BEHAVIOURAL PHARMACOLOGY	10.1097/FBP.0000000000000145
2015	Pelli, M; Connaughton, VP	Chronic exposure to environmentally-relevant concentrations of fluoxetine (Prozac) decreases survival, increases abnormal behaviors, and delays predator escape responses in guppies	CHEMOSPHERE	10.1016/j.chemosphere.2015.06.033

2015	Idalencio, R; Kalichak, F; Rosa, JGS; de Oliveira, TA; Koakoski, G; Gusso, D; de Abreu, MS; Giacomini, ACV; Barcellos, HHD; Piato, AL; Barcellos, LJG	Waterborne Risperidone Decreases Stress Response in Zebrafish	PLOS ONE	10.1371/journal.pone.0140800
2015	Sebire, M; Davis, JE; Hatfield, R; Winberg, S; Katsiadaki, I	Prozac affects stickleback nest quality without altering androgen, spiggin or aggression levels during a 21-day breeding test	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2015.09.009
2015	Greaney, NE; Mannion, KL; Dziewczynski, TL	Signaling on Prozac: altered audience effects on male-male interactions after fluoxetine exposure in Siamese fighting fish	BEHAVIORAL ECOLOGY AND SOCIOBIOLOGY	10.1007/s00265-015-2005-y
2015	Smith, MS; Booth, NJ; Peterson, BC; Stephens, WS; Goudie, CA; Simco, BA	Analysis of Short-Term Cortisol Stress Response in Channel Catfish by Anesthetization with Metomidate Hydrochloride and Tricaine Methanesulfonate	JOURNAL OF AQUATIC ANIMAL HEALTH	10.1080/08997659.2015.1047537
2015	Abreu, MS; Giacomini, ACV; Koakoski, G; Oliveira, TA; Gusso, D; Baldisserotto, B; Barcellos, LJG	Effects of waterborne fluoxetine on stress response and osmoregulation in zebrafish	ENVIRONMENTAL TOXICOLOGY AND PHARMACOLOGY	10.1016/j.etap.2015.09.001
2015	Eisenreich, BR; Szalda-Petree, A	Behavioral effects of fluoxetine on aggression and associative learning in Siamese fighting fish (*Betta splendens*)	BEHAVIOURAL PROCESSES	10.1016/j.beproc.2015.10.008
2015	Weinert, NC; Volpato, J; Costa, A; Antunes, RR; de Oliveira, AC; Mattoso, CRS; Saito, ME	Hematology of Nile tilapia (*Oreochromis niloticus*) subjected to anesthesia and anticoagulation protocols	SEMINA-CIENCIAS AGRARIAS	10.5433/1679-0359.2015v36n6Supl2p4237
2015	Silvia, R; Torres, T; Martins, R; Santos, MM	Toxicity screening of Diclofenac, Propranolol, Sertraline and Simvastatin using *Danio rerio* and *Paracentrotus lividus* embryo bioassays	ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY	10.1016/j.ecoenv.2015.01.008
2015	Magno, LDP; Fontes, A; Goncalves, BMN; Gouveia, A	Reprint of "Pharmacological study of the light/dark preference test in zebrafish (*Danio rerio*): Waterborne administration"	PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR	10.1016/j.pbb.2015.11.001
2014	Steenbergen, PJ; Bardine, N	Antinociceptive effects of buprenorphine in zebrafish larvae: An alternative for rodent models to study pain and nociception?	APPLIED ANIMAL BEHAVIOUR SCIENCE	10.1016/j.applanim.2013.12.001
2014	Cosenza, GR; Claudiano, GS; Marcusso, PF; Eto, SF; Manrique, WG; Loureiro, BA; Shimada, MT; Salvador, R; Moraes, JRE; Moraes, FR	Influence of glyceryl guaiacolate ether on anesthetics in tilapia compared to benzocaine and eugenol	REVISTA MVZ CORDOBA	10.21897/rmvz.114
2014	Collymore, C; Tolwani, A; Lieggi, C; Rasmussen, S	Efficacy and Safety of 5 Anesthetics in Adult Zebrafish (*Danio rerio*)	JOURNAL OF THE AMERICAN ASSOCIATION FOR LABORATORY ANIMAL SCIENCE	
2014	Trickler, WJ; Guo, XQ; Cuevas, E; Ali, SF; Paule, MG; Kanungo, J	Ketamine attenuates cytochrome p450 aromatase gene expression and estradiol-17 beta levels in zebrafish early life stages	JOURNAL OF APPLIED TOXICOLOGY	10.1002/jat.2888
2014	Valenca-Silva, G; Braz, MG; Barreto, RE; Salvadori, DMF; Volpato, GL	Low Dose of the Anesthetic Propofol Does Not Induce Genotoxic or Mutagenic Effects in Nile Tilapia	TRANSACTIONS OF THE AMERICAN FISHERIES SOCIETY	10.1080/00028487.2013.856814

2014	Nunes, B; Barbosa, AR; Antunes, SC; Goncalves, F	Combination effects of anticholinesterasics in acetylcholinesterase of a fish species: effects of a metallic compound, an organophosphate pesticide, and a pharmaceutical drug	ENVIRONMENTAL SCIENCE AND POLLUTION RESEARCH	10.1007/s11356-014-2584-6
2014	Craig, PM; Trudeau, VL; Moon, TW	Profiling Hepatic micrornas in Zebrafish: Fluoxetine Exposure Mimics a Fasting Response That Targets AMP-Activated Protein Kinase (AMPK)	PLOS ONE	10.1371/journal.pone.0095351
2014	Yang, M; Qiu, WH; Chen, JS; Zhan, J; Pan, CY; Lei, XJ; Wu, MH	Growth inhibition and coordinated physiological regulation of zebrafish (*Danio rerio*) embryos upon sublethal exposure to antidepressant amitriptyline	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2013.12.029
2014	Weinberger, J; Klaper, R	Environmental concentrations of the selective serotonin reuptake inhibitor fluoxetine impact specific behaviors involved in reproduction, feeding and predator avoidance in the fish *Pimephales promelas* (fathead minnow)	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2013.10.012
2014	Hedgespeth, ML; Nilsson, PA; Berglund, O	Ecological implications of altered fish foraging after exposure to an antidepressant pharmaceutical	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2013.12.011
2014	Bisesi, JH; Bridges, W; Klaine, SJ	Reprint of: Effects of the antidepressant venlafaxine on fish brain serotonin and predation behavior	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2014.02.015
2014	Olsen, KH; Ask, K; Olsen, H; Porsch-Hallstrom, I; Hallgren, S	Reprint of "Effects of the SSRI citalopram on behaviours connected to stress and reproduction in Endler guppy, *Poecilia wingei*"	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2014.02.011
2014	Connors, KA; Valenti, TW; Lawless, K; Sackerman, J; Onaivi, ES; Brooks, BW; Gould, GG	Similar anxiolytic effects of agonists targeting serotonin 5-HT1A or cannabinoid CB receptors on zebrafish behavior in novel environments	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2013.12.005
2014	Galus, M; Rangarajan, S; Lai, A; Shaya, L; Balshine, S; Wilson, JY	Effects of chronic, parental pharmaceutical exposure on zebrafish (*Danio rerio*) offspring	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2014.01.016
2014	Minter, LJ; Bailey, KM; Harms, CA; Lewbart, GA; Posner, LP	The efficacy of alfaxalone for immersion anesthesia in koi carp (*Cyprinus carpio*)	VETERINARY ANAESTHESIA AND ANALGESIA	10.1111/vaa.12113
2014	Parker, MO; Brock, AJ; Sudwarts, A; Brennan, CH	Atomoxetine reduces anticipatory responding in a 5-choice serial reaction time task for adult zebrafish	PSYCHOPHARMACOLOGY	10.1007/s00213-014-3439-z
2014	Fraser, TWK; Mayer, I; Skjaeraasen, JE; Hansen, T; Fjelldal, PG	The effect of triploidy on the efficacy and physiological response to anesthesia with MS 222 and isoeugenol in Atlantic salmon post-smolts	AQUACULTURE INTERNATIONAL	10.1007/s10499-014-9751-0
2014	Wagner, KA; Woodley, CM; Seaburg, AG; Skalski, JR; Eppard, MB	Physiological Stress Responses to Prolonged Exposure to MS-222 and Surgical Implantation in Juvenile Chinook Salmon	NORTH AMERICAN JOURNAL OF FISHERIES MANAGEMENT	10.1080/02755947.2014.926303
2014	Gao, Y; Li, DP; Peng, XZ; Tang, R	Effects of low-voltage constant direct current on plasma biochemical profiles and gene expression levels in crucian carp *Carassius carassius*	FISHERIES SCIENCE	10.1007/s12562-014-0792-0

2014	Best, C; Melnyk-Lamont, N; Gesto, M; Vijayan, MM	Environmental levels of the antidepressant venlafaxine impact the metabolic capacity of rainbow trout	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2014.06.014
2014	Mitjana, O; Bonastre, C; Insua, D; Falceto, MV; Esteban, J; Josa, A; Espinosa, E	The efficacy and effect of repeated exposure to 2-phenoxyethanol, clove oil and tricaine methanesulphonate as anesthetic agents on juvenile Angelfish (*Pterophyllum scalare*)	AQUACULTURE	10.1016/j.aquaculture.2014.07.013
2014	Margiotta-Casaluci, L; Owen, SF; Cumming, RI; de Polo, A; Winter, MJ; Panter, GH; Rand-Weaver, M; Sumpter, JP	Quantitative Cross-Species Extrapolation between Humans and Fish: The Case of the Anti-Depressant Fluoxetine	PLOS ONE	10.1371/journal.pone.0110467
2014	Forsatkar, MN; Nematollahi, MA; Amiri, BM; Huang, WB	Fluoxetine inhibits aggressive behaviour during parental care in male fighting fish (*Betta splendens*, Regan)	ECOTOXICOLOGY	10.1007/s10646-014-1345-0
2014	Melnyk-Lamont, N; Best, C; Gesto, M; Vijayan, MM	The Antidepressant Venlafaxine Disrupts Brain Monoamine Levels and Neuroendocrine Responses to Stress in Rainbow Trout	ENVIRONMENTAL SCIENCE & TECHNOLOGY	10.1021/es504331n
2014	Spears, J; Kamunde, C; Stevens, ED	Effect of TRIS and Bicarbonate as Buffers on Anesthetic Efficacy of Tricaine Methane Sulfonate in Zebrafish (*Danio rerio*)	ZEBRAFISH	10.1089/zeb.2014.0975
2014	Oda, A; Bailey, KM; Lewbart, GA; Griffith, EH; Posner, LP	Physiologic and biochemical assessments of koi (*Cyprinus carpio*) following immersion in propofol	JAVMA-JOURNAL OF THE AMERICAN VETERINARY MEDICAL ASSOCIATION	10.2460/javma.245.11.1286
2014	Abreu, Murilo Sander de; Koakoski, Gessi; Ferreira, Daiane; Oliveira, Thiago Acosta; Rosa, João Gabriel Santos; Gusso, Darlan; Giacomini, Ana Cristina Varrone; Piato, Angelo Luis; Barcello, Leonardo Jose´ Gil	Diazepam and Fluoxetine Decrease the Stress Response in Zebrafish	PLOS ONE	10.1371/journal.pone.0103232
2013	GholipourKanani, H; Ahadizadeh, S	Use of propofol as an anesthetic and its efficacy on some hematological values of ornamental fish *Carassius auratus*	SPRINGERPLUS	10.1186/2193-1801-2-76
2013	Brodin, T; Fick, J; Jonsson, M; Klaminder, J	Dilute Concentrations of a Psychiatric Drug Alter Behavior of Fish from Natural Populations	SCIENCE	10.1126/science.1226850
2013	Barry, MJ	Effects of fluoxetine on the swimming and behavioural responses of the Arabian killifish	ECOTOXICOLOGY	10.1007/s10646-012-1036-7
2013	Kanani, HG; Soltani, M; Mirzargar, SS	Effect of tricainemethanesulfonate (MS222), clove oil and electro-anaesthesia on respiratory burst activity in whole blood and serum alternative complement response in rainbow trout (*Oncorhynchus mykiss*), during the narcosis stage	FISH & SHELLFISH IMMUNOLOGY	10.1016/j.fsi.2012.11.021
2013	Lopez-Bellido, R; Barreto-Valer, K; Rodriguez, RE	Expression of tachykinin receptors (tacr1a and tacr1b) in zebrafish: influence of cocaine and opioid receptors	JOURNAL OF MOLECULAR ENDOCRINOLOGY	10.1530/JME-12-0199

2013	Kanungo, J; Cuevas, E; Ali, SF; Paule, MG	Ketamine induces motor neuron toxicity and alters neurogenic and proneural gene expression in zebrafish	JOURNAL OF APPLIED TOXICOLOGY	10.1002/jat.1751
2013	Galus, M; Kirischian, N; Higgins, S; Purdy, J; Chow, J; Rangaranjan, S; Li, HX; Metcalfe, C; Wilson, JY	Chronic, low concentration exposure to pharmaceuticals impacts multiple organ systems in zebrafish	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2012.12.021
2013	Bauquier, SH; Greenwood, J; Whittem, T	Evaluation of the sedative and anaesthetic effects of five different concentrations of alfaxalone in goldfish, *Carassius auratus*	AQUACULTURE	10.1016/j.aquaculture.2013.02.021
2013	Bailey, KM; Hempstead, JE; Tobias, JR; Borst, LB; Clode, AB; Posner, LP	Evaluation of the effects of tricaine methanesulfonate on retinal structure and function in koi carp (*Cyprinus carpio*)	JAVMA-JOURNAL OF THE AMERICAN VETERINARY MEDICAL ASSOCIATION	10.2460/javma.242.11.1578
2013	Lopez-Bellido, R; Barreto-Valer, K; Rodriguez, RE	Substance p mrna expression during zebrafish development: influence of mu opioid receptor and cocaine	NEUROSCIENCE	10.1016/j.neuroscience.2013.03.022
2013	de Assis, HCS; Simmons, DBD; Zamora, JM; Lado, WE; Al-Ansari, AM; Sherry, JP; Blais, JM; Metcalfe, CD; Trudeau, VL	Estrogen-like Effects in Male Goldfish Co-exposed to Fluoxetine and 17 Alpha-Ethinylestradiol	ENVIRONMENTAL SCIENCE & TECHNOLOGY	10.1021/es3044888
2013	Christiansen, HE; Gee, LP; Mesa, MG	Anesthesia of Juvenile Pacific Lampreys with MS-222, BENZOAK, AQUI-S 20E, and Aquacalm	NORTH AMERICAN JOURNAL OF FISHERIES MANAGEMENT	10.1080/02755947.2012.754807
2013	Alsop, D; Wood, CM	Metal and pharmaceutical mixtures: Is ion loss the mechanism underlying acute toxicity and widespread additive toxicity in zebrafish?	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2013.05.021
2013	Morato-Fernandes, J; Tavares, RA; Rocha, CB; Pouey, JLOF; Piedras, SRN	Benzocaine and clove oil as anesthetics for pejerrey (*Odontesthes bonariensis*) fingerlings	ARQUIVO BRASILEIRO DE MEDICINA VETERINARIA E ZOOTECNIA	10.1590/S0102-09352013000500024
2013	de Padua, SB; Neto, JD; Sakabe, R; Claudiano, GD; Chagas, EC; Pilarski, F	Hematologic variables in tambaquis anesthetized with clove oil and benzocaine	PESQUISA AGROPECUARIA BRASILEIRA	10.1590/S0100-204X2013000800056
2013	Brandao, FP; Rodrigues, S; Castro, BB; Goncalves, F; Antunes, SC; Nunes, B	Short-term effects of neuroactive pharmaceutical drugs on a fish species: Biochemical and behavioural effects	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2013.10.005
2013	Zivna, D; Plhalova, L; Praskova, E; Stepanova, S; Siroka, Z; Sevcikova, M; Blahova, J; Bartoskova, M; Marsalek, P; Skoric, M; Svobodova, Z	Oxidative stress parameters in fish after subchronic exposure to acetylsalicylic acid	NEUROENDOCRINOLOGY LETTERS	
2013	Posner, LP; Scott, GN; Law, JM	Repeated exposure of goldfish (*Carassius auratus*) to tricaine methanesulfonate (MS-222)	JOURNAL OF ZOO AND WILDLIFE MEDICINE	10.1638/2012-0151R1.1

2012	Praskova, E; Zivna, D; Stepanova, S; Sevcikova, M; Blahova, J; Marsalek, P; Siroka, Z; Voslarova, E; Svobodova, Z	Acute toxicity of acetylsalicylic acid to juvenile and embryonic stages of *Danio rerio*	NEUROENDOCRINOLOGY LETTERS	
2012	Thomas, MA; Joshi, PP; Klaper, RD	Gene-class analysis of expression patterns induced by psychoactive pharmaceutical exposure in fathead minnow (*Pimephales promelas*) indicates induction of neuronal systems	COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY C-TOXICOLOGY & PHARMACOLOGY	10.1016/j.cbpc.2011.05.014
2012	Valenti, TW; Gould, GG; Berninger, JP; Connors, KA; Keele, NB; Prosser, KN; Brooks, BW	Human Therapeutic Plasma Levels of the Selective Serotonin Reuptake Inhibitor (SSRI) Sertraline Decrease Serotonin Reuptake Transporter Binding and Shelter-Seeking Behavior in Adult Male Fathead Minnows	ENVIRONMENTAL SCIENCE & TECHNOLOGY	10.1021/es204164b
2012	Richendrfer, H; Pelkowski, SD; Colwill, RM; Creton, R	On the edge: Pharmacological evidence for anxiety-related behavior in zebrafish larvae	BEHAVIOURAL BRAIN RESEARCH	10.1016/j.bbr.2011.11.041
2012	Wu, MM; Khan, IA; Dasmahapatra, AK	Valproate-induced teratogenesis in Japanese rice fish (*Oryzias latipes*) embryogenesis	COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY C-TOXICOLOGY & PHARMACOLOGY	10.1016/j.cbpc.2012.01.003
2012	Ali, S; Champagne, DL; Richardson, MK	Behavioral profiling of zebrafish embryos exposed to a panel of 60 water-soluble compounds	BEHAVIOURAL BRAIN RESEARCH	10.1016/j.bbr.2011.11.020
2012	Kohlert, JG; Mangan, BP; Kodra, C; Drako, L; Long, E; Simpson, H	Decreased aggressive and locomotor behaviors in betta splendens after exposure to fluoxetine	PSYCHOLOGICAL REPORTS	10.2466/02.13.PR0.110.1.51-62
2012	Winder, VL; Pennington, PL; Hurd, MW; Wirth, EF	Fluoxetine effects on sheepshead minnow (*Cyprinodon variegatus*) locomotor activity	JOURNAL OF ENVIRONMENTAL SCIENCE AND HEALTH PART B-PESTICIDES FOOD CONTAMINANTS AND AGRICULTURAL WASTES	10.1080/03601234.2012.607767
2012	Barba-Escobedo, PA; Gould, GG	Visual social preferences of lone zebrafish in a novel environment: strain and anxiolytic effects	GENES BRAIN AND BEHAVIOR	10.1111/j.1601-183X.2012.00770.x
2012	Overturf, MD; Overturf, CL; Baxter, D; Hala, DN; Constantine, L; Venables, B; Huggett, DB	Early Life-Stage Toxicity of Eight Pharmaceuticals to the Fathead Minnow, *Pimephales promelas*	ARCHIVES OF ENVIRONMENTAL CONTAMINATION AND TOXICOLOGY	10.1007/s00244-011-9723-6
2012	Madureira, TV; Rocha, MJ; Cruzeiro, C; Rodrigues, I; Monteiro, RAF; Rocha, E	The toxicity potential of pharmaceuticals found in the Douro River estuary (Portugal): Evaluation of impacts on fish liver, by histopathology, stereology, vitellogenin and CYP1A immunohistochemistry, after sub-acute exposures of the zebrafish model	ENVIRONMENTAL TOXICOLOGY AND PHARMACOLOGY	10.1016/j.etap.2012.02.007

2012	Trushenski, JT; Bowker, JD; Gause, BR; Mulligan, BL	Chemical and Electrical Approaches to Sedation of Hybrid Striped Bass: Induction, Recovery, and Physiological Responses to Sedation	TRANSACTIONS OF THE AMERICAN FISHERIES SOCIETY	10.1080/00028487.2012.664603
2012	Delbon, MC; Paiva, MJTR	Eugenol in tilapia juvenile: concentrations and successive administrations	BOLETIM DO INSTITUTO DE PESCA	
2012	Tuckey, NPL; Forgan, LG	A rapid and simple fluorometric method for quantifying isoeugenol in seawater and in plasma and white muscle from Australasian snapper (*Pagrus auratus*)	FOOD CHEMISTRY	10.1016/j.foodchem.2012.02.069
2012	Togunde, OP; Oakes, KD; Servos, MR; Pawliszyn, J	Determination of Pharmaceutical Residues in Fish Bile by Solid-Phase Microextraction Couple with Liquid Chromatography-Tandem Mass Spectrometry (LC/MS/MS)	ENVIRONMENTAL SCIENCE & TECHNOLOGY	10.1021/es203758n
2012	Rodrigues, S; Antunes, SC; Brandao, FP; Castro, BB; Goncalves, F; Nunes, B	Effects of anticholinesterase drugs on biomarkers and behavior of pumpkinseed, *Lepomis gibbosus* (Linnaeus, 1758)	JOURNAL OF ENVIRONMENTAL MONITORING	10.1039/c2em30033h
2012	Fredricks, KT; Meinertz, JR; Ambrose, RD; Jackan, LM; Wise, JK; Gaikowski, MP	Feeding Response of Sport Fish after Electrical Immobilization, Chemical Sedation, or Both	NORTH AMERICAN JOURNAL OF FISHERIES MANAGEMENT	10.1080/02755947.2012.686955
2012	Kim, P; Park, Y; Ji, K; Seo, J; Lee, S; Choi, K; Kho, Y; Park, J; Choi, K	Effect of chronic exposure to acetaminophen and lincomycin on Japanese medaka (*Oryzias latipes*) and freshwater cladocerans *Daphnia magna* and *Moina macrocopa*, and potential mechanisms of endocrine disruption	CHEMOSPHERE	10.1016/j.chemosphere.2012.04.006
2012	Hu, XQ; Li, Y; Hu, ZY; Rudd, JA; Ling, SC; Jiang, FZ; Davies, H; Fang, MR	The alteration of 5-HT2A and 5-HT2C receptors is involved in neuronal apoptosis of goldfish cerebellum following traumatic experience	NEUROCHEMISTRY INTERNATIONAL	10.1016/j.neuint.2012.04.022
2012	Dzieweczynski, TL; Hebert, OL	Fluoxetine alters behavioral consistency of aggression and courtship in male Siamese fighting fish, *Betta splendens*	PHYSIOLOGY & BEHAVIOR	10.1016/j.physbeh.2012.06.007
2012	Maaswinkel, H; Zhu, LQ; Weng, W	The immediate and the delayed effects of buspirone on zebrafish (*Danio rerio*) in an open field test: A 3-D approach	BEHAVIOURAL BRAIN RESEARCH	10.1016/j.bbr.2012.07.014
2012	Gause, BR; Trushenski, JT; Bowzer, JC; Bowker, JD	Efficacy and Physiological Responses of Grass Carp to Different Sedation Techniques: I. Effects of Various Chemicals on Sedation and Blood Chemistry	NORTH AMERICAN JOURNAL OF AQUACULTURE	10.1080/15222055.2012.691013
2012	de Souza, RAR; de Carvalho, CVA; Nunes, FF; Scopel, BR; Guarizi, JD; Tsuzuki, MY	Comparative effect of benzocaine, menthol and eugenol as anesthetics for juvenile fat snook	BOLETIM DO INSTITUTO DE PESCA	
2012	Bittencourt, F; Souza, BE; Boscolo, WR; Rorato, RR; Feiden, A; Neu, DH	Benzocaine and eugenol as anesthetics for golden fish (*Carassius auratus*)	ARQUIVO BRASILEIRO DE MEDICINA VETERINARIA E ZOOTECNIA	10.1590/S0102-09352012000600028
2012	da Rocha, MA; Grumadas, CES; Ribeiro, ELD; Mizubuti, IY; Ludovico, A; Constatino, C	Determination of the optimal dose of benzocaine hydrochloride in anesthesia of tilapia (*Oreochromis niloticus*)	SEMINA-CIENCIAS AGRARIAS	10.5433/1679-0359.2012v33n6p2403

2012	Garcia, SN; Foster, M; Constantine, LA; Huggett, DB	Field and laboratory fish tissue accumulation of the anti-convulsant drug carbamazepine	ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY	10.1016/j.ecoenv.2012.07.013
2011	Park, IS; Park, SJ; Gil, HW; Nam, YK; Kim, DS	Anesthetic effects of clove oil and lidocaine-hcl on marine medaka (*Oryzias dancena*)	LAB ANIMAL	10.1038/laban0211-45
2011	Sanchez-Vazquez, FJ; Terry, MI; Felizardo, VO; Vera, LM	Daily Rhythms of Toxicity and Effectiveness of Anesthetics (MS222 and Eugenol) in Zebrafish (*Danio Rerio*)	CHRONOBIOLOGY INTERNATIONAL	10.3109/07420528.2010.538105
2011	Mathur, P; Lau, B; Guo, S	Conditioned place preference behavior in zebrafish	NATURE PROTOCOLS	10.1038/nprot.2010.201
2011	Cachat, J; Stewart, A; Utterback, E; Hart, P; Gaikwad, S; Wong, K; Kyzar, E; Wu, N; Kalueff, AV	Three-Dimensional Neurophenotyping of Adult Zebrafish Behavior	PLOS ONE	10.1371/journal.pone.0017597
2011	Li, ZH; Zlabek, V; Velisek, J; Grabic, R; Machova, J; Kolarova, J; Li, P; Randak, T	Acute toxicity of carbamazepine to juvenile rainbow trout (*Oncorhynchus mykiss*): Effects on antioxidant responses, hematological parameters and hepatic EROD	ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY	10.1016/j.ecoenv.2010.09.008
2011	Holmberg, A; Fogel, J; Albertsson, E; Fick, J; Brown, JN; Paxeus, N; Forlin, L; Johnsson, JI; Larsson, DGJ	Does waterborne citalopram affect the aggressive and sexual behaviour of rainbow trout and guppy?	JOURNAL OF HAZARDOUS MATERIALS	10.1016/j.jhazmat.2011.01.055
2011	Matsche, MA	Evaluation of tricaine methanesulfonate (MS-222) as a surgical anesthetic for Atlantic Sturgeon *Acipenser oxyrinchus oxyrinchus*	JOURNAL OF APPLIED ICHTHYOLOGY	10.1111/j.1439-0426.2011.01714.x
2011	Di Marco, P; Petochi, T; Longobardi, A; Priori, A; Finoia, MG; Donadelli, V; Corsalini, I; Marino, G	Efficacy of tricaine methanesulphonate, clove oil and medetomidine-ketamine and their side effects on the physiology of sturgeon hybrid *Acipenser naccarii x Acipenser baerii*	JOURNAL OF APPLIED ICHTHYOLOGY	10.1111/j.1439-0426.2011.01701.x
2011	Grossman, L; Stewart, A; Gaikwad, S; Utterback, E; Wu, N; DiLeo, J; Frank, K; Hart, P; Howard, H; Kalueff, AV	Effects of piracetam on behavior and memory in adult zebrafish	BRAIN RESEARCH BULLETIN	10.1016/j.brainresbull.2011.02.008
2011	Schultz, MM; Painter, MM; Bartell, SE; Logue, A; Furlong, ET; Werner, SL; Schoenfuss, HL	Selective uptake and biological consequences of environmentally relevant antidepressant pharmaceutical exposures on male fathead minnows	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2011.03.011
2011	Gebauer, DL; Pagnussat, N; Piato, AL; Schaefer, IC; Bonan, CD; Lara, DR	Effects of anxiolytics in zebrafish: Similarities and differences between benzodiazepines, buspirone and ethanol	PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR	10.1016/j.pbb.2011.04.021
2011	Kanani, HG; Mirzargar, SS; Soltani, M; Ahmadi, M; Abrishamifar, A; Bahonar, A; Yousefi, P	Anesthetic effect of tricaine methanesulfonate, clove oil and electroanesthesia on lysozyme activity of *Oncorhynchus mykiss*	IRANIAN JOURNAL OF FISHERIES SCIENCES	
2011	Weber, RA; Perez-Maceira, JJ; Peleteiro, JB; Garcia-Martin, L; Aldegunde, M	Effects of acute exposure to 2-phenoxyethanol, clove oil, MS-222, and metomidate on primary and secondary stress responses in Senegalese sole (*Solea senegalensis* Kaup 1858)	AQUACULTURE	10.1016/j.aquaculture.2011.08.029

2011	Madureira, TV; Rocha, MJ; Cruzeiro, C; Galante, MH; Monteiro, RAF; Rocha, E	The toxicity potential of pharmaceuticals found in the Douro River estuary (Portugal): Assessing impacts on gonadal maturation with a histopathological and stereological study of zebrafish ovary and testis after sub-acute exposures	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2011.06.017
2011	Khor, BS; Jamil, MFA; Adenan, MI; Shu-Chien, AC	Mitragynine Attenuates Withdrawal Syndrome in Morphine-Withdrawn Zebrafish	PLOS ONE	10.1371/journal.pone.0028340
2010	Li, ZH; Zlabek, V; Velisek, J; Grabic, R; Machova, J; Randak, T	Modulation of antioxidant defence system in brain of rainbow trout (*Oncorhynchus mykiss*) after chronic carbamazepine treatment	COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY C-TOXICOLOGY & PHARMACOLOGY	10.1016/j.cbpc.2009.09.006
2010	Li, ZH; Velisek, J; Zlabek, V; Grabic, R; Machova, J; Kolarova, J; Randak, T	Hepatic antioxidant status and hematological parameters in rainbow trout, *Oncorhynchus mykiss*, after chronic exposure to carbamazepine	CHEMICO-BIOLOGICAL INTERACTIONS	10.1016/j.cbi.2009.09.009
2010	Kucuk, S	Efficacy of tricaine on *Peocilia latipinna* at different temperatures and concentrations	AFRICAN JOURNAL OF BIOTECHNOLOGY	10.5897/AJB09.1353
2010	Hampel, M; Alonso, E; Aparicio, I; Bron, JE; Santos, JL; Taggart, JB; Leaver, MJ	Potential physiological effects of pharmaceutical compounds in Atlantic salmon (*Salmo salar*) implied by transcriptomic analysis	ENVIRONMENTAL SCIENCE AND POLLUTION RESEARCH	10.1007/s11356-009-0282-6
2010	Villeneuve, DL; Garcia-Reyero, N; Martinovic, D; Mueller, ND; Cavallin, JE; Durhan, EJ; Makynen, EA; Jensen, KM; Kahl, MD; Blake, LS; Perkins, EJ; Ankley, GT	I. Effects of a dopamine receptor antagonist on fathead minnow, *Pimephales promelas*, reproduction	ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY	10.1016/j.ecoenv.2009.09.007
2010	Villeneuve, DL; Garcia-Reyero, N; Martinovic, D; Mueller, ND; Cavallin, JE; Durhan, EJ; Makynen, EA; Jensen, KM; Kahl, MD; Blake, LS; Perkins, EJ; Ankley, GT	II: Effects of a dopamine receptor antagonist on fathead minnow dominance behavior and ovarian gene expression in the fathead minnow and zebrafish	ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY	10.1016/j.ecoenv.2009.09.018
2010	Li, ZH; Zlabek, V; Grabic, R; Velisek, J; Machova, J; Randak, T	Enzymatic alterations and RNA/DNA ratio in intestine of rainbow trout, *Oncorhynchus mykiss*, induced by chronic exposure to carbamazepine	ECOTOXICOLOGY	10.1007/s10646-010-0468-1
2010	Al-Hamdani, AH; Ebrahim, SK; Mohammad, FK	Experimental Xylazine-Ketamine Anesthesia in the Common Carp (*Cyprinus carpio*)	JOURNAL OF WILDLIFE DISEASES	10.7589/0090-3558-46.2.596
2010	Li, ZH; Zlabek, V; Velisek, J; Grabic, R; Machova, J; Randak, T	Physiological condition status and muscle-based biomarkers in rainbow trout (*Oncorhynchus mykiss*), after long-term exposure to carbamazepine	JOURNAL OF APPLIED TOXICOLOGY	10.1002/jat.1482
2010	Okamura, D; de Araujo, FG; Rosa, PVE; de Freitas, RTF; Murgas, LDS; Cesar, MP	Effect of benzocaine concentration and fish size on anesthesia and recovery in Nile tilapia	REVISTA BRASILEIRA DE ZOOTECNIA-	10.1590/S1516-35982010000500005

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2010	Gladden, JN; Brainard, BM; Shelton, JL; Camus, AC; Divers, SJ	Evaluation of isoeugenol for anesthesia in koi carp (*Cyprinus carpio*)	AMERICAN JOURNAL OF VETERINARY RESEARCH	10.2460/ajvr.71.8.859
2010	Nassef, M; Matsumoto, S; Seki, M; Khalil, F; Kang, IJ; Shimasaki, Y; Oshima, Y; Honjo, T	Acute effects of triclosan, diclofenac and carbamazepine on feeding performance of Japanese medaka fish (*Oryzias latipes*)	CHEMOSPHERE	10.1016/j.chemosphere.2010.04.073
2010	Wong, K; Stewart, A; Gilder, T; Wu, N; Frank, K; Gaikwad, S; Suciu, C; Dileo, J; Utterback, E; Chang, K; Grossman, L; Cachat, J; Kalueff, AV	Modeling seizure-related behavioral and endocrine phenotypes in adult zebrafish	BRAIN RESEARCH	10.1016/j.brainres.2010.06.012
2010	Mennigen, JA; Sassine, J; Trudeau, VL; Moon, TW	Waterborne fluoxetine disrupts feeding and energy metabolism in the goldfish *Carassius auratus*	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2010.07.022
2010	Huang, WC; Hsieh, YS; Chen, IH; Wang, CH; Chang, HW; Yang, CC; Ku, TH; Yeh, SR; Chuang, YJ	Combined Use of MS-222 (Tricaine) and Isoflurane Extends Anesthesia Time and Minimizes Cardiac Rhythm Side Effects in Adult Zebrafish	ZEBRAFISH	10.1089/zeb.2010.0653
2010	van den Brandhof, EJ; Montforts, M	Fish embryo toxicity of carbamazepine, diclofenac and metoprolol	ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY	10.1016/j.ecoenv.2010.08.031
2010	Mennigen, JA; Lado, WE; Zamora, JM; Duarte-Guterman, P; Langlois, VS; Metcalfe, CD; Chang, JP; Moon, TW; Trudeau, VL	Waterborne fluoxetine disrupts the reproductive axis in sexually mature male goldfish, *Carassius auratus*	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2010.08.016
2010	Feng, GP; Zhuang, P; Zhang, LZ; Liu, JY; Feng, GP; Zhuang, P	Blood Biochemical Responses of Juvenile Chinese Sturgeon (*Acipenser sinensis*) to Anesthetic Tricaine Methanesulfonate	2010 4TH INTERNATIONAL CONFERENCE ON BIOINFORMATICS AND BIOMEDICAL ENGINEERING (ICBBE 2010)	10.1109/ICBBE.2010.5518114
2010	Painter, Meghan M., Buerkley, Megan A., Matthew L. Julius Alan M. Vajda David O. Norris Larry B. Barber Edward T. Furlong Melissa M. Schultz Heiko L. Schoenfuss	Antidepressants at environmentally relevant concentrations affect predator avoidance behavior of larval fathead minnows (*Pimephales promelas*)	ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY	10.1897/08-556.1
2010	Oggier, DM; Weisbrod, CI; Stoller, AM; Zenker, AK; Fent, K	Effects of Diazepam on Gene Expression and Link to Physiological Effects in Different Life Stages in Zebrafish *Danio rerio*	ENVIRONMENTAL SCIENCE & TECHNOLOGY	10.1021/es100980r

2009	Minagh, E; Hernan, R; O'Rourke, K; Lyng, FM; Davoren, M	Aquatic ecotoxicity of the selective serotonin reuptake inhibitor sertraline hydrochloride in a battery of freshwater test species	ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY	10.1016/j.ecoenv.2008.05.002
2009	Kiessling, A; Johansson, D; Zahl, IH; Samuelsen, OB	Pharmacokinetics, plasma cortisol and effectiveness of benzocaine, MS-222 and isoeugenol measured in individual dorsal aorta-cannulated Atlantic salmon (*Salmo salar*) following bath administration	AQUACULTURE	10.1016/j.aquaculture.2008.09.037
2009	Kim, JW; Ishibashi, H; Yamauchi, R; Ichikawa, N; Takao, Y; Hirano, M; Koga, M; Arizono, K	Acute toxicity of pharmaceutical and personal care products on freshwater crustacean (*Thamnocephalus platyurus*) and fish (*Oryzias latipes*)	JOURNAL OF TOXICOLOGICAL SCIENCES	10.2131/jts.34.227
2009	Winder, VL; Sapozhnikova, Y; Pennington, PL; Wirth, EF	Effects of fluoxetine exposure on serotonin-related activity in the sheepshead minnow (*Cyprinodon variegatus*) using LC/MS/MS detection and quantitation	COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY C-TOXICOLOGY & PHARMACOLOGY	10.1016/j.cbpc.2008.12.008
2009	Tuckey, NPL; Forster, ME; Gieseg, SP	Lipid Oxidation Is Inhibited by Isoeugenol Exposure in Chinook Salmon (*Oncorhynchus Tshawytscha*) Fillets during Storage at 15 degrees C	JOURNAL OF FOOD SCIENCE	10.1111/j.1750-3841.2009.01135.x
2009	Okamoto, MH; Tesser, MB; Louzada, LR; dos Santos, RA; Sampaio, LA	Benzocaine and eugenol as anaesthetics for pompano juvenile *Trachinotus marginatus*	CIENCIA RURAL	10.1590/S0103-84782008005000100
2009	Newby, NC; Wilkie, MP; Stevens, ED	Morphine uptake, disposition, and analgesic efficacy in the common goldfish (*Carassius auratus*)	CANADIAN JOURNAL OF ZOOLOGY	10.1139/Z09-023
2009	Sattari, A; Mirzargar, SS; Abrishamifar, A; Lourakzadegan, R; Bahonar, A; Mousavi, HE; Niasari, A	Comparison of Electroanesthesia with Chemical Anesthesia (MS222 and Clove Oil) in Rainbow Trout (*Oncorhynchus mykiss*) using Plasma Cortisol and Glucose Responses as Physiological Stress Indicators	ASIAN JOURNAL OF ANIMAL AND VETERINARY ADVANCES	
2009	Zahl, IH; Kiessling, A; Samuelsen, OB; Hansen, MK	Anaesthesia of Atlantic cod (*Gadus morhua*) - Effect of pre-anaesthetic sedation, and importance of body weight, temperature and stress	AQUACULTURE	10.1016/j.aquaculture.2009.06.019
2009	Meinertz, JR; Schreier, TM	Depletion of isoeugenol residues from the fillet tissue of AQUI-S (TM) exposed rainbow trout (*Oncorhynchus mykiss*)	AQUACULTURE	10.1016/j.aquaculture.2009.08.022
2009	Velasco-Santamaria, Y; Palacios-Ruiz, C; Cruz-Casallas, P	Efficiency of 2-phenoxyethanol, benzocaine, quinaldine and tricaine methasulphonate as an anesthesia for pirapitinga (*Piaractus brachypomus*) fingerlings and juvenile fishes	REVISTA MVZ CORDOBA	
2009	Bencan, Z; Sledge, D; Levin, ED	Buspirone, chlordiazepoxide and diazepam effects in a zebrafish model of anxiety	PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR	10.1016/j.pbb.2009.07.009
2009	Valenti, TW; Perez-Hurtado, P; Chambliss, CK; Brooks, BW	Aquatic toxicity of sertraline to *Pimephales promelas* at environmentally relevant surface water pH	ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY	10.1897/08-546.1

2009	Nassef, M; Matsumoto, S; Seki, M; Kang, IJ; Moroishi, J; Shimasaki, Y; Oshima, Y	Pharmaceuticals and Personal Care Products Toxicity to Japanese Medaka Fish (*Oryzias latipes*)	JOURNAL OF THE FACULTY OF AGRICULTURE KYUSHU UNIVERSITY	
2009	Lister, A; Regan, C; Van Zwol, J; Van Der Kraak, G	Inhibition of egg production in zebrafish by fluoxetine and municipal effluents: A mechanistic evaluation	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2009.04.011
2009	Li, ZH; Zlabek, V; Velisek, J; Grabic, R; Machova, J; Randak, T	Responses of antioxidant status and Na+–K+-atpase activity in gill of rainbow trout, *Oncorhynchus mykiss*, chronically treated with carbamazepine	CHEMOSPHERE	10.1016/j.chemosphere.2009.10.031
2008	Henry, TB; Black, MC	Acute and chronic toxicity of fluoxetine (selective serotonin reuptake inhibitor) in western mosquitofish	ARCHIVES OF ENVIRONMENTAL CONTAMINATION AND TOXICOLOGY	10.1007/s00244-007-9018-0
2008	Nakamura, Y; Yamamoto, H; Sekizawa, J; Kondo, T; Hirai, N; Tatarazako, N	The effects of pH on fluoxetine in Japanese medaka (*Oryzias latipes*): Acute toxicity in fish larvae and bioaccumulation in juvenile fish	CHEMOSPHERE	10.1016/j.chemosphere.2007.06.089
2008	Antunes, MIP; Spurio, RS; Godoi, DA; Grumadas, CE; da Rocha, MA	Benzocaine hydrochloride anesthesia in carp (*Cyprinus carpio*)	SEMINA-CIENCIAS AGRARIAS	10.5433/1679-0359.2008v29n1p151
2008	Zhou, SN; Oakes, KD; Servos, MR; Pawliszyn, J	Application of solid-phase microextraction for *in vivo* laboratory and field sampling of pharmaceuticals in fish	ENVIRONMENTAL SCIENCE & TECHNOLOGY	10.1021/es8001162
2008	Gaworecki, KM; Klaine, SJ	Behavioral and biochemical responses of hybrid striped bass during and after fluoxetine exposure	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2008.04.011
2008	Nunes, B; Gaio, AR; Carvalho, F; Guilhermino, L	Behaviour and biomarkers of oxidative stress in *Gambusia holbrooki* after acute exposure to widely used pharmaceuticals and a detergent	ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY	10.1016/j.ecoenv.2007.12.006
2008	Bencan, Z; Levin, ED	The role of alpha 7 and alpha 4 beta 2 nicotinic receptors in the nicotine-induced anxiolytic effect in zebrafish	PHYSIOLOGY & BEHAVIOR	10.1016/j.physbeh.2008.07.009
2008	Paterson, G; Metcalfe, CD	Uptake and depuration of the anti-depressant fluoxetine by the Japanese medaka (*Oryzias latipes*)	CHEMOSPHERE	10.1016/j.chemosphere.2008.08.022
2007	Levin, ED; Bencan, Z; Cerutti, DT	Anxiolytic effects of nicotine in zebrafish	PHYSIOLOGY & BEHAVIOR	10.1016/j.physbeh.2006.08.026
2007	Triebskorn, R; Casper, H; Scheil, V; Schwaiger, J	Ultrastructural effects of pharmaceuticals (carbamazepine, clofibric acid, metoprolol, diclofenac) in rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*)	ANALYTICAL AND BIOANALYTICAL CHEMISTRY	10.1007/s00216-006-1033-x
2007	Bosworth, BG; Small, BC; Gregory, D; Kim, J; Black, S; Jerrett, A	Effects of rested-harvest using the anesthetic AQUI-S (TM) on channel catfish, *Ictalurus punctatus*, physiology and fillet quality	AQUACULTURE	10.1016/j.aquaculture.2006.10.035
2007	Sink, TD; Strange, RJ; Sawyers, RE	Clove oil used at lower concentrations is less effective than MS-222 at reducing cortisol stress responses in anesthetized rainbow trout	NORTH AMERICAN JOURNAL OF FISHERIES MANAGEMENT	10.1577/M05-103.1

2007	Kim, Y; Choi, K; Jung, JY; Park, S; Kim, PG; Park, J	Aquatic toxicity of acetaminophen, carbamazepine, cimetidine, diltiazem and six major sulfonamides, and their potential ecological risks in Korea	ENVIRONMENT INTERNATIONAL	10.1016/j.envint.2006.11.017
2007	Burgess, HA; Granato, M	Sensorimotor gating in larval zebrafish	JOURNAL OF NEUROSCIENCE	10.1523/JNEUROSCI.0615-07.2007
2007	Cordova, MS; Braun, CB	The use of anesthesia during evoked potential audiometry in goldfish (*Carassius auratus*)	BRAIN RESEARCH	10.1016/j.brainres.2007.03.055
2007	Olsvik, PA; Lie, KK; Hevroy, EM	Do anesthetics and sampling strategies affect transcription analysis of fish tissues?	BMC MOLECULAR BIOLOGY	10.1186/1471-2199-8-48
2007	Hilvarsson, A; Halldorsson, HP; Granmo, A	Medetomidine as a candidate antifoulant: Sublethal effects on juvenile turbot (*Psetta maxima* L.)	AQUATIC TOXICOLOGY	10.1016/j.aquatox.2007.04.008
2007	Stanley, JK; Ramirez, AJ; Chambliss, CK; Brooks, BW	Enantiospecific sublethal effects of the antidepressant fluoxetine to a model aquatic vertebrate and invertebrate	CHEMOSPHERE	10.1016/j.chemosphere.2007.04.080
2007	Lynn, SE; Egar, JM; Walker, BG; Sperry, TS; Ramenofsky, M	Fish on Prozac: a simple, noninvasive physiology laboratory investigating the mechanisms of aggressive behavior in *Betta splendens*	ADVANCES IN PHYSIOLOGY EDUCATION	10.1152/advan.00024.2007
2006	Crosby, TC; Hill, JE; Watson, CA; Yanong, RPE; Strange, R	Effects of tricaine methanesulfonate, hypno, metomidate, quinaldine, and salt on plasma cortisol levels following acute stress in threespot gourami *Trichogaster trichopterus*	JOURNAL OF AQUATIC ANIMAL HEALTH	10.1577/H05-026.1
2006	Meinertz, JR; Greseth, SL; Schreier, TM; Bernardy, JA; Gingerich, WH	Isoeugenol concentrations in rainbow trout (*Oncorhynchus mykiss*) skin-on fillet tissue after exposure to AQUI-S (TM) at different temperatures, durations, and concentrations	AQUACULTURE	10.1016/j.aquaculture.2005.09.028
2006	Palic, D; Herolt, DM; Andreasen, CB; Menzel, BW; Roth, JA	Anesthetic efficacy of tricaine methanesulfonate, metomidate and eugenol: Effects on plasma cortisol concentration and neutrophil function in fathead minnows (*Pimephales promelas* Rafinesque, 1820)	AQUACULTURE	10.1016/j.aquaculture.2005.11.004
2006	Cotter, PA; Rodnick, KJ	Differential effects of anesthetics on electrical properties of the rainbow trout (*Oncorhynchus mykiss*) heart	COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY A-MOLECULAR & INTEGRATIVE PHYSIOLOGY	10.1016/j.cbpa.2006.06.001
2006	Bolasina, SN	Cortisol and hematological response in Brazilian codling, *Urophycis brasiliensis* (Pisces, Phycidae) subjected to anesthetic treatment	AQUACULTURE INTERNATIONAL	10.1007/s10499-006-9055-0
2006	van der Ven, K; Keil, D; Moens, LN; Van Hummelen, P; van Remortel, P; Maras, M; De Coen, W	Effects of the antidepressant mianserin in zebrafish: Molecular markers of endocrine disruption	CHEMOSPHERE	10.1016/j.chemosphere.2006.03.079

2005	Small, BC; Chatakondi, N	Routine measures of stress are reduced in mature channel catfish during and after AQUI-S anesthesia and recovery	NORTH AMERICAN JOURNAL OF AQUACULTURE	10.1577/FA04-028.1
2005	Nunes, B; Carvalho, F; Guilhermino, L	Acute toxicity of widely used pharmaceuticals in aquatic species: *Gambusia holbrooki*, *Artemia parthenogenetica* and *Tetraselmis chuii*	ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY	10.1016/j.ecoenv.2004.08.010
2005	Marsic-Lucic, J; Mladineo, I; Tudor, M	Comparative effectiveness of 2-phenoxyethanol and Propiscin as anesthetics for juvenile sea bass *Dicentrarchus labrax* L.	AQUACULTURE INTERNATIONAL	10.1007/s10499-005-9005-2
2005	Dietrich, GJ; Kowalski, R; Wojtczak, M; Dobosz, S; Goryczko, K; Ciereszko, A	Motility parameters of rainbow trout (*Oncorhynchus mykiss*) spermatozoa in relation to sequential collection of milt, time of post-mortem storage and anesthesia	FISH PHYSIOLOGY AND BIOCHEMISTRY	10.1007/s10695-005-3527-4
2004	Levine, J; Panchalingam, K; McClure, RJ; Gershon, S; Pettegrew, JW	Effects of acetyl-L-carnitine and myo-inositol on high-energy phosphate and membrane phospholipid metabolism in zebra fish: A 31P-NMR-spectroscopy study	JOURNAL OF AFFECTIVE DISORDERS	10.1023/a:1022849430947
2004	Bressler, K; Ron, B	Effect of anesthetics on stress and the innate immune system of gilthead seabream (*Sparus aurata*)	ISRAELI JOURNAL OF AQUACULTURE-BAMIDGEH	10.46989/001c.20367
2004	Davis, KB; Griffin, BR	Physiological responses of hybrid striped bass under sedation by several anesthetics	AQUACULTURE	10.1016/j.aquaculture.2003.09.018
2004	Foran, CM; Weston, J; Slattery, M; Brooks, BW; Huggett, DB	Reproductive assessment of Japanese medaka (*Oryzias latipes*) following a four-week fluoxetine (SSRI) exposure	ARCHIVES OF ENVIRONMENTAL CONTAMINATION AND TOXICOLOGY	10.1007/s00244-003-3042-5
2004	Palmer, LM; Mensinger, AF	Effect of the anesthetic tricaine (MS-222) on nerve activity in the anterior lateral line of the oyster toadfish, *Opsanus tau*	JOURNAL OF NEUROPHYSIOLOGY	10.1152/jn.01151.2003
2004	Holcomb, M; Woolsey, J; Cloud, JG; Ingermann, RL	Effects of clove oil, tricaine, and CO2 on gamete quality in steelhead and white sturgeon	NORTH AMERICAN JOURNAL OF AQUACULTURE	10.1577/A03-027.1
2004	Arkhipchuk, VV; Goncharuk, VV; Chernykh, VP; Maloshtan, LN; Gritsenko, IS	Use of a complex approach for assessment of metamizole sodium and acetylsalicylic acid toxicity, genotoxicity and cytotoxicity	JOURNAL OF APPLIED TOXICOLOGY	10.1002/jat.1027
2004	Ciltas, A; Erdogan, O; Hisar, O; Kocaman, EM	Inhibition effect of some chemical Anesthetics and hypothermia on the activity of glucose 6-phosphate dehydrogenase from rainbow trout (*Oncorhynchus mykiss*) erythrocytes *in vivo*	TURKISH JOURNAL OF VETERINARY & ANIMAL SCIENCES	
2003	Gontijo, AMDC; Barreto, RE; Speit, G; Reyes, VAV; Volpato, GL; Salvadori, DMF	Anesthesia of fish with benzocaine does not interfere with comet assay results	MUTATION RESEARCH-GENETIC TOXICOLOGY AND ENVIRONMENTAL MUTAGENESIS	10.1016/S1383-5718(02)00276-0

2003	Small, BC	Anesthetic efficacy of metomidate and comparison of plasma cortisol responses to tricaine methanesulfonate, quinaldine and clove oil anesthetized channel catfish *Ictalurus punctatus*	AQUACULTURE	10.1016/S0044-8486(02)00302-2
2003	Hansen, MK; Nymoen, U; Horsberg, TE	Pharmacokinetic and pharmacodynamic properties of metomidate in turbot (*Scophthalmus maximus*) and halibut (*Hippoglossus hippoglossus*)	JOURNAL OF VETERINARY PHARMACOLOGY AND THERAPEUTICS	10.1046/j.1365-2885.2003.00454.x
2003	Ferrari, B; Paxeus, N; Lo Giudice, R; Pollio, A; Garric, J	Ecotoxicological impact of pharmaceuticals found in treated wastewaters: study of carbamazepine, clofibric acid, and diclofenac	ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY	10.1016/S0147-6513(02)00082-9
2003	Cuesta, A; Esteban, MA; Meseguer, J	Effects of different stressor agents on gilthead seabream natural cytotoxic activity	FISH & SHELLFISH IMMUNOLOGY	10.1016/S1050-4648(03)00022-6
2003	Brooks, BW; Turner, PK; Stanley, JK; Weston, JJ; Glidewell, EA; Foran, CM; Slattery, M; La Point, TW; Huggett, DB	Waterborne and sediment toxicity of fluoxetine to select organisms	CHEMOSPHERE	10.1016/S0045-6535(03)00103-6
2002	Wagner, E; Arndt, R; Hilton, B	Physiological stress responses, egg survival and sperm motility for rainbow trout broodstock anesthetized with clove oil, tricaine methanesulfonate or carbon dioxide	AQUACULTURE	10.1016/S0044-8486(01)00878-X
2001	Sladky, KK; Swanson, CR; Stoskopf, MK; Loomis, MR; Lewbart, GA	Comparative efficacy of tricaine methanesulfonate and clove oil for use as anesthetics in red pacu (*Piaractus brachypomus*)	AMERICAN JOURNAL OF VETERINARY RESEARCH	10.2460/ajvr.2001.62.337
2001	Gomes, LC; Chippari-Gomes, AR; Lopes, NP; Roubach, R; Araujo-Lima, CARM	Efficacy of benzocaine as an anesthetic in juvenile tambaqui *Colossoma macropomum*	JOURNAL OF THE WORLD AQUACULTURE SOCIETY	10.1111/j.1749-7345.2001.tb00470.x
2001	Zhdanova, IV; Wang, SY; Leclair, OU; Danilova, NP	Melatonin promotes sleep-like state in zebrafish	Brain Research Interactive	10.1016/S0006-8993(01)02444-1
2000	Davidson, GW; Davie, PS; Young, G; Fowler, RT	Physiological responses of rainbow trout *Oncorhynchus mykiss* to crowding and anesthesia with AQUI-S (TM)	JOURNAL OF THE WORLD AQUACULTURE SOCIETY	10.1111/j.1749-7345.2000.tb00704.x
1999	Stehly, GR; Gingerich, WH	Evaluation of AQUI-S (TM) (efficacy and minimum toxic concentration) as a fish anaesthetic sedative for public aquaculture in the United States	AQUACULTURE RESEARCH	10.1046/j.1365-2109.1999.00339.x
1999	Smith, DA; Smith, SA; Holladay, SD	Effect of previous exposure to tricaine methanesulfonate on time to anesthesia in hybrid tilapias	JOURNAL OF AQUATIC ANIMAL HEALTH	10.1577/1548-8667(1999)011<0183:EOPETT>2.0.CO;2
1998	Auperin, B; Goardon, L; Quemeneur, A; Thomas, JL; Aubin, J; Valotaire, C; Rouger, Y; Maisse, G	Preliminary study on the use of AQUI-S (R) as anesthetic for handling and sampling of rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*).	BULLETIN FRANCAIS DE LA PECHE ET DE LA PISCICULTURE	10.1051/kmae:1998006

1997	MacAvoy, SE; Zaepfel, RC	Effects of tricaine methanesulfonate (MS-222) on hematocrit: First field measurements on blacknose dace	TRANSACTIONS OF THE AMERICAN FISHERIES SOCIETY	10.1577/1548-8659(1997)126<0500:EOT-MMO>2.3.CO;2
1997	Munday, PL; Wilson, SK	Comparative efficacy of clove oil and other chemicals in anaesthetization of *Pomacentrus amboinensis*, a coral reef fish	JOURNAL OF FISH BIOLOGY	10.1111/j.1095-8649.1997.tb01532.x
1995	RODRIGUEZ-GUTIERREZ, M; ESQUIVEL-HERRERA, A	Evaluation of the repeated use of xylocaine as anesthetic for the handling of breeding carp (*Cyprinus carpio*)	AQUACULTURE	10.1016/0044-8486(94)00280-2
1995	OLSEN, YA; EINARSDOTTIR, IE; NILSSEN, KJ	Metomidate anesthesia in atlantic salmon, S*almo salar*, prevents plasma-cortisol increase during stress	AQUACULTURE	10.1016/0044-8486(95)00008-P
1995	MASSEE, KC; RUST, MB; HARDY, RW; STICKNEY, RR	The effectiveness of tricaine, quinaldine sulfate and metomidate as anesthetics for larval fish	AQUACULTURE	10.1016/0044-8486(95)00057-9
1994	ARINC, E; SEN, A	In-vivo effects of the anesthetic, benzocaine, on liver microsomal cytochrome-p450 and mixed-function oxidase activities of gilthead seabream (*Sparus aurata*)	COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY C-PHARMACOLOGY TOXICOLOGY & ENDOCRINOLOGY	10.1016/1367-8280(94)90068-X
1994	UEDA, I; TATARA, T; CHIOU, JS; KRISHNA, PR; KAMAYA, H	Structure-selective anesthetic action of steroids - anesthetic potency and effects on lipid and protein	ANESTHESIA AND ANALGESIA	10.1213/00000539-199404000-00018
1994	LOSEY, GS; HUGIE, DM	Prior anesthesia impairs a chemically mediated fright response in a gobiid fish	JOURNAL OF CHEMICAL ECOLOGY	10.1007/BF02066229
1993	MALMSTROM, T; SALTE, R; GJOEN, HM; LINSETH, A	A practical evaluation of metomidate and MS-222 as anesthetics for atlantic halibut (*Hippoglossus-hippoglosus* l)	AQUACULTURE	10.1016/0044-8486(93)90403-L
1993	ROSS, RM; BACKMAN, TWH; BENNETT, RM	Evaluation of the anesthetic metomidate for the handling and transport of juvenile american shad	PROGRESSIVE FISH-CULTURIST	10.1577/1548-8640(1993)055<0236:EOTAMF>2.3.CO;2
1991	SPOTTE, S; BUBUCIS, PM; ANDERSON, G	Plasma-cortisol response of seawater-adapted mummichogs (*Fundulus heteroclitus*) during deep MS-222 anesthesia	ZOO BIOLOGY	10.1002/zoo.1430100110
1991	BARHAM, WT; SCHOONBEE, HJ	A comparison of the effects of alternating-current electronarcosis, rectified current electronarcosis and chemical anesthesia on the blood physiology of the fresh-water bream *Oreochromis mossambicus* (peters) .2. The effect on hematocrit, hemoglobin concentration, red-cell count, mean cell-volume, mean cell hemoglobin and mean cell hemoglobin concentration	COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY A-PHYSIOLOGY	10.1016/0300-9629(91)90516-F
1991	THOMAS, P; ROBERTSON, L	Plasma-cortisol and glucose stress responses of red drum (*Sciaenops ocellatus*) to handling and shallow-water stressors and anesthesia with MS-222, quinaldine sulfate and metomidate	AQUACULTURE	10.1016/0044-8486(91)90140-3

1991	GILDERHUS, PA; LEMM, CA; WOODS, LC	Benzocaine as an anesthetic for striped bass	PROGRESSIVE FISH-CULTURIST	10.1577/1548-8640(1991)053<0105:BAAAFS>2.3.CO;2
1991	HARRINGTON, AJ; RUSSELL, KA; SINGER, TD; BALLANTYNE, JS	The effects of tricaine methanesulfonate (MS-222) on plasma nonesterified fatty-acids in rainbow-trout, *Oncorhynchus-mykiss*	LIPIDS	10.1007/BF02535630
1990	GILDERHUS, PA	Benzocaine as a fish anesthetic - efficacy and safety for spawning-phase salmon	PROGRESSIVE FISH-CULTURIST	10.1577/1548-8640(1990)052<0189:BAAFAE>2.3.CO;2
1989	IWAMA, GK; MCGEER, JC; PAWLUK, MP	The effects of 5 fish anesthetics on acid-base balance, hemato-crit, blood-gases, cortisol, and adrenaline in rainbow-trout	CANADIAN JOURNAL OF ZOOLOGY-REVUE CANADIENNE DE ZOOLOGIE	10.1139/z89-294
1989	GABRYELAK, T; ZALESNA, G; ROCHE, H; PERES, G	The effect of MS-222 an anesthetic on the peroxide metabolism enzymes in erythrocytes of fresh-water and marine fish species	COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY C-PHARMACOLOGY TOXICOLOGY & ENDOCRINOLOGY	10.1016/0742-8413(89)90193-X
1983	LIMSUWAN, C; GRIZZLE, JM; PLUMB, JA	Etomidate as an anesthetic for fish - its toxicity and efficacy	TRANSACTIONS OF THE AMERICAN FISHERIES SOCIETY	10.1577/1548-8659(1983)112<544:EAAAFF>2.0.CO;2
1982	AMEND, DF; GOVEN, BA; ELLIOT, DG	Etomidate - effective dosages for a new fish anesthetic	TRANSACTIONS OF THE AMERICAN FISHERIES SOCIETY	10.1577/1548-8659(1982)111<337:E>2.0.CO;2
1981	BILLARD, R	Effect of some fish anesthetics on gamete survival during arti-ficial-insemination of rainbow-trout	PROGRESSIVE FISH-CULTURIST	10.1577/1548-8659(1981)43[72:EOSFAO]2.0.CO;2
1979	SMIT, GL; HATTINGH, J; BURGER, AP	Hematological assessment of the effects of the anesthetic MS-222 in natural and neutralized form in 3 freshwater-fish species - interspecies differences	JOURNAL OF FISH BIOLOGY	10.1111/j.1095-8649.1979.tb03672.x
1979	SMIT, GL; HATTINGH, J; BURGER, AP	Hematological assessment of the effects of the anesthetic MS-222 in natural and neutralized form in 3 freshwater-fish species - hemoglobin electrophoresis, ATP levels and corpuscular fragility curves	JOURNAL OF FISH BIOLOGY	10.1111/j.1095-8649.1979.tb03674.x

Table A 1.3. Environmental levels reported for neuroactive pharmaceuticals detected in surface water samples from freshwater and brackish/marine environments.

Pharmaceutical	Environment	Surface water origin	Average concentration (µg/l)	Maximum concentration (µg/L)	Maximum type (from source)	Original reference	Search	DOI
Amitriptyline	Freshwater	River/Stream	0.025500	0.071	Max	Baker & Kasprzyk-Hordern (2013)	der Beek (2016)	10.1002/etc.3339
Amitriptyline	Freshwater	River/Stream	0.002250	0.0025	Max	Aminot et al. (2015)	This study	10.1007/s00216-015-9017-3
Amitriptyline	Freshwater	River/Stream	0.000610	0.00073	Max	Kondor et al. (2020)	This study	10.1016/j.envpol.2020.114893
Amitriptyline	Freshwater	River/Stream	0.018667	0.022	Max	Thomas et al. (2014)	This study	10.1111/jawr.12164
Amitriptyline	Estuarine/Marine	Estuary		0.0006	Max	Klosterhaus et al. (2013)	der Beek (2016)	10.1002/etc.3339
Amitriptyline	Estuarine/Marine	Estuary		0.0064	Max	Ziarrusta et al. (2015)	This study	10.1007/s00216-015-9224-y
Amitriptyline	Estuarine/Marine	Estuary	0.0003	0.0003	Average	Aminot et al. (2016)	This study	10.1016/j.marchem.2016.05.010
Amitriptyline	Estuarine/Marine	Sea or Ocean		0.026	Max	Togola & Budzinski (2008)	der Beek (2016)	10.1002/etc.3339
Bromazepam	Freshwater	River/Stream	0.0011	0.0011	Max	Aminot et al. (2015)	This study	10.1007/s00216-015-9017-3
Bromazepam	Freshwater	River/Stream	0.0438	0.134	Single value	Togola et al. (2008)	der Beek (2016)	10.1002/etc.3339
Bromazepam	Freshwater	River/Stream	0.0953	0.32	Max	Fick et al. (2017)	This study	10.1016/j.chemosphere.2017.02.126
Bromazepam	Freshwater	River/Stream	0.0005	0.00073	Max	Brieudes et al. (2017)	This study	10.1016/j.jchromb.2016.07.016
Bromazepam	Estuarine/Marine	Estuary		0.356	Max	Sadezky et al. (2008)	der Beek (2016)	10.1002/etc.3339
Bupropion	Freshwater	River/Stream	0.08075	0.14	Average	Ferrer & Thurman (2012)	der Beek (2016)	10.1002/etc.3339
Bupropion	Freshwater	River/Stream	0.00058	0.0006	Max	Kondor et al. (2020)	This study	10.1016/j.envpol.2020.114893

Bupropion	Estuarine/Marine	Sea or Ocean	0.0024	0.0024	Single value	Bjorlenius et al. (2018)	This study	10.1016/j.scitotenv.2018.03.276
Carbamazepine	Freshwater	Lake		0.67	Max	Han tran et al. (2013)	This study	10.1002/clen.201300021
Carbamazepine	Freshwater	Lake	0.101013063	8.053	Single value	Narbaitz et al. (2013)	der Beek (2016)	10.1002/etc.3339
Carbamazepine	Freshwater	Lake	0.01235	0.031	Max	Pascual-Aguilar et al. (2013)	This study	10.1016/j.jhazmat.2013.07.052
Carbamazepine	Freshwater	Lake	0.00223	0.01	Max	Ferguson et al. (2013)	This study	10.1016/j.scitotenv.2013.04.024
Carbamazepine	Freshwater	Lake		0.014	Max	Moschet et al. (2013)	This study	10.1021/es304484w
Carbamazepine	Freshwater	River/Stream	0.127	0.127	Single value	Glaser et al. (2020)	This study	10.1002/hyp.13909
Carbamazepine	Freshwater	River/Stream	0.0621	0.151	Max	Aminot et al. (2015)	This study	10.1007/s00216-015-9017-3
Carbamazepine	Freshwater	River/Stream		0.14	Max	Vystavna et al. (2012)	This study	10.1007/s10661-012-2811-x
Carbamazepine	Freshwater	River/Stream		0.094	Max	Radović et al. (2014)	This study	10.1007/s10661-014-4092-z
Carbamazepine	Freshwater	River/Stream	0.0245	0.044	Max	López et al. (2014)	This study	10.1007/s11356-014-3187-y
Carbamazepine	Freshwater	River/Stream	0.409569826	11.56123	Single value	Loos et al. (2008)	der Beek (2016)	10.1002/etc.3339
Carbamazepine	Freshwater	River/Stream	0.428333333	1.65	Average	Matongo et al. (2015)[a]	This study	10.1007/s11356-015-4217-0
Carbamazepine	Freshwater	River/Stream		0.0165	Max	Lalović et al. (2017)	This study	10.1007/s11356-017-9748-0
Carbamazepine	Freshwater	River/Stream		0.0165	Max	Lalović et al. (2017)	This study	10.1007/s11356-017-9748-0
Carbamazepine	Freshwater	River/Stream	0.011386552	0.0229	Max	Milić et al. (2018)	This study	10.1007/s11356-018-1401-z
Carbamazepine	Freshwater	River/Stream	0.995	3.24	Average	Matongo et al. (2015)[b]	This study	10.1016/j.chemosphere.2015.03.093
Carbamazepine	Freshwater	River/Stream	0.2183	0.2183	Average	Archer et al. (2017)	This study	10.1016/j.chemosphere.2017.01.101
Carbamazepine	Freshwater	River/Stream	0.28	0.354	Average	Fernandes et al. (2020)	This study	10.1016/j.chemosphere.2019.124729
Carbamazepine	Freshwater	River/Stream		0.48	Max	Park and Jeon (2021)	This study	10.1016/j.chemosphere.2020.128014

Carbamazepine	Freshwater	River/Stream	0.016444444	0.026	Max	Fonseca et al. (2020)	This study	10.1016/j.envint.2020.106004
Carbamazepine	Freshwater	River/Stream	0.0772	0.498	Max	Kondor et al. (2020)	This study	10.1016/j.envpol.2020.114893
Carbamazepine	Freshwater	River/Stream	0.00151	0.0088	Max	Hossain 2018	This study	10.1016/j.envres.2018.04.030
Carbamazepine	Freshwater	River/Stream	0.0177	0.0318	Max	Brieudes et al. (2017)	This study	10.1016/j.jchromb.2016.07.016
Carbamazepine	Freshwater	River/Stream	0.0013	0.002	Max	Čelić et al. (2021)	This study	10.1016/j.jhazmat.2020.124102
Carbamazepine	Freshwater	River/Stream	4.8475	12.96	Max	Salvatierra-stamp et al. (2018)	This study	10.1016/j.micro.2018.04.012
Carbamazepine	Freshwater	River/Stream	0.012294118	0.04	Max	Chițescu et al. (2015)	This study	10.1016/j.scitotenv.2015.06.010
Carbamazepine	Freshwater	River/Stream	0.01475	0.038	Average	Andreu et al. (2016)	This study	10.1016/j.scitotenv.2015.08.007
Carbamazepine	Freshwater	River/Stream	0.050621935	0.21	Max	Fairbairn et al. (2016)	This study	10.1016/j.scitotenv.2016.02.056
Carbamazepine	Freshwater	River/Stream		0.214	Max	Paíga et al. (2016)	This study	10.1016/j.scitotenv.2016.08.089
Carbamazepine	Freshwater	River/Stream	0.09	0.276	Max	Rivera-Jaimes et al. (2018)	This study	10.1016/j.scitotenv.2017.09.134
Carbamazepine	Freshwater	River/Stream	0.033566667	0.0631	Average	Skees et al. (2018)	This study	10.1016/j.scitotenv.2018.03.060
Carbamazepine	Freshwater	River/Stream		1.763	Max	Park et al. (2018)	This study	10.1016/j.scitotenv.2018.05.081
Carbamazepine	Freshwater	River/Stream	0.809	5.32	Max	Česen et al. (2019)	This study	10.1016/j.scitotenv.2018.09.238
Carbamazepine	Freshwater	River/Stream	0.040844444	0.1123	Average	Čelić et al. (2019)	This study	10.1016/j.scitotenv.2018.10.290
Carbamazepine	Freshwater	River/Stream	0.015133799	0.06743285	Max	Su et al. (2020)	This study	10.1016/j.scitotenv.2019.134525
Carbamazepine	Freshwater	River/Stream	0.0603	1.81	Max	Yang et al. (2021)	This study	10.1016/j.scitotenv.2020.144080
Carbamazepine	Freshwater	River/Stream	0.017428571	0.073	Max	Boix et al. (2015)	This study	10.1016/j.talanta.2014.08.005
Carbamazepine	Freshwater	River/Stream	0.021976667	0.02256	Average	Brieudes et al. (2016)	This study	10.1016/j.talanta.2015.06.073

Carbamazepine	Freshwater	River/Stream	0.0139	0.295	Average	Arlos et al. (2015)	This study	10.1016/j.watres.2014.11.008
Carbamazepine	Freshwater	River/Stream	0.501295337	0.501295337	Average	Silva et al. (2020)	This study	10.1039/D0AY00426J
Carbamazepine	Freshwater	River/Stream		0.026	Max	Bernot et al. (2019)	This study	10.1080/00288330.2018.1457062
Carbamazepine	Freshwater	River/Stream		0.025	Max	Chițescu and Nicolau (2014)	This study	10.1080/02772248.2015.1005092
Carbamazepine	Freshwater	River/Stream	0.073	0.146	Max	Stamatis et al. (2013)	This study	10.1080/03067319.2013.814121
Carbamazepine	Freshwater	River/Stream	0.053755	0.531	Max	Camilleri et al. (2014)	This study	10.1080/03067319.2014.983494
Carbamazepine	Freshwater	River/Stream		0.0033	Max	Arbeláez et al. (2015)	This study	10.1080/03067319.2015.1055474
Carbamazepine	Freshwater	River/Stream	0.00465	0.0068	Max	Kiguchi et al. (2018)	This study	10.1080/03067319.2019.1637425
Carbamazepine	Freshwater	River/Stream	0.228111111	0.652	Max	Thomas et al. (2014)	This study	10.1111/jawr.12164
Carbamazepine	Freshwater	River/Stream	4.811683333	17.3452	Max	Vumazonke et al. (2020)	This study	10.3390/ijerph17114067
Carbamazepine	Freshwater	River/Stream	0.120935	0.64331	Max	Burcea et al. (2020)	This study	10.3390/su12231019 7
Carbamazepine	Freshwater	River/Stream		0.243	Max	Vilimanovic et al. (2020)	This study	10.3934/environsci.2020019
Carbamazepine	Freshwater	River/Stream		0.0154	Max	Petre et al. (2016)	This study	https://revistadechimie.ro/Articles.asp?ID=5113
Carbamazepine	Estuarine/Marine	Estuary	0.022506373	0.997	Max	Sadezky et al. (2008)	der Beek (2016)	10.1002/etc.3339
Carbamazepine	Estuarine/Marine	Estuary	0.0123	0.018	Average	Aminot et al. (2016)	This study	10.1016/j.marchem.2016.05.010
Carbamazepine	Estuarine/Marine	Estuary		0.1064	Max	Anim et al. (2020)	This study	10.1016/j.marpolbul.2020.111014
Carbamazepine	Estuarine/Marine	Estuary	0.00135	0.00339	Max	Zhao et al. (2015)	This study	10.1016/j.scitotenv.2015.06.055
Carbamazepine	Estuarine/Marine	Estuary	0.000890323	0.005	Average	Čelić et al. (2019)	This study	10.1016/j.scitotenv.2018.10.290
Carbamazepine	Estuarine/Marine	Estuary	0.00586	0.00586	Max	Ariel et al. (2021)	This study	10.3390/ijerph18030943

Carbamazepine	Estuarine/Marine	Sea or Ocean	0.000155	0.00071	Max	Bueno et al. (2016)	This study	10.1007/s11356-014-3796-5
Carbamazepine	Estuarine/Marine	Sea or Ocean	0.000966667	0.0018	Max	Roveri et al. (2020)	This study	10.1007/s11356-020-10316-y
Carbamazepine	Estuarine/Marine	Sea or Ocean	0.036564	0.1096	Max	Ali et al. (2017)	This study	10.1016/j.chemosphere.2017.02.095
Carbamazepine	Estuarine/Marine	Sea or Ocean	0.02364375	0.119	Single value	Wille et al. (2010)	der Beek (2016)	10.1002/etc.3339
Carbamazepine	Estuarine/Marine	Sea or Ocean	0.0003	0.0005	Max	Čelić et al. (2021)	This study	10.1016/j.jhazmat.2020.124102
Carbamazepine	Estuarine/Marine	Sea or Ocean	0.03725	0.321	Max	Claessens et al. (2013)	This study	10.1016/j.marpolbul.2013.03.039
Carbamazepine	Estuarine/Marine	Sea or Ocean		0.157	Max	Nodler et al. (2014)	This study	10.1016/j.marpolbul.2014.06.024
Carbamazepine	Estuarine/Marine	Sea or Ocean	0.000828813	0.00463	Max	Bayen et al. (2016)	This study	10.1016/j.marpolbul.2016.06.105
Carbamazepine	Estuarine/Marine	Sea or Ocean	0.55125	1.41	Max	McEneff et al. (2014)	This study	10.1016/j.scitotenv.2013.12.123
Carbamazepine	Estuarine/Marine	Sea or Ocean	0.000582353	0.0014	Max	Alygizakis et al. (2016)	This study	10.1016/j.scitotenv.2015.09.145
Carbamazepine	Estuarine/Marine	Sea or Ocean	0.003582857	0.019	Max	Bjorlenius et al. (2018)	This study	10.1016/j.scitotenv.2018.03.276
Citalopram	Freshwater	Lake	3.052726842	8	Single value	Fick et al. (2009)	der Beek (2016)	10.1002/etc.3339
Citalopram	Freshwater	River/Stream	0.043857143	0.126	Max	López et al. (2014)	This study	10.1007/s11356-014-3187-y
Citalopram	Freshwater	River/Stream	0.0679	0.0679	Average	Fernandes et al. (2020)	This study	10.1016/j.chemosphere.2019.124729
Citalopram	Freshwater	River/Stream	0.009666667	0.017	Average	Giebułtowicz and Nałęcz-Jawecki (2014)	This study	10.1016/j.ecoenv.2014.02.020
Citalopram	Freshwater	River/Stream	0.00161	0.015	Max	Kondor et al. (2020)	This study	10.1016/j.envpol.2020.114893
Citalopram	Freshwater	River/Stream	4.737923077	76	Single value	Fick et al. (2009)	der Beek (2016)	10.1002/etc.3339
Citalopram	Freshwater	River/Stream	0.00429	0.00752	Max	Brieudes et al. (2017)	This study	10.1016/j.jchromb.2016.07.016
Citalopram	Freshwater	River/Stream		0.0289	Max	Paíga et al. (2016)	This study	10.1016/j.scitotenv.2016.08.089

Citalopram	Freshwater	River/Stream	0.0346	0.0951	Average	Skees et al. (2018)	This study	10.1016/j.scitotenv.2018.03.060
Citalopram	Freshwater	River/Stream	0.032871429	0.1018	Average	Čelić et al. (2019)	This study	10.1016/j.scitotenv.2018.10.290
Citalopram	Freshwater	River/Stream	0.00346	0.00369	Average	Brieudes et al. (2016)	This study	10.1016/j.talanta.2015.06.073
Citalopram	Freshwater	River/Stream	0.0612	0.0790	Max	Thomas et al. (2014)	This study	10.1111/jawr.12164
Citalopram	Estuarine/Marine	Estuary	0.026527778	0.0925	Max	Fernandez-Rubio et al. (2019)	This study	10.1016/j.chemosphere.2019.02.041
Citalopram	Estuarine/Marine	Estuary		0.0026	Max	Anim et al. (2020)	This study	10.1016/j.marpolbul.2020.111014
Citalopram	Estuarine/Marine	Estuary	0.0031	0.0031	Average	Čelić et al. (2019)	This study	10.1016/j.scitotenv.2018.10.290
Citalopram	Estuarine/Marine	Sea or Ocean	0.0002	0.0002	Max	Roveri et al. (2020)	This study	10.1007/s11356-020-10316-y
Citalopram	Estuarine/Marine	Sea or Ocean	0.004	0.004	Single value	Gros et al. (2012)	der Beek (2016)	10.1002/etc.3339
Citalopram	Estuarine/Marine	Sea or Ocean		0.027	Max	Nodler et al. (2014)	This study	10.1016/j.marpolbul.2014.06.024
Citalopram	Estuarine/Marine	Sea or Ocean	0.003190909	0.008	Max	Alygizakis et al. (2016)	This study	10.1016/j.scitotenv.2015.09.145
Citalopram	Estuarine/Marine	Sea or Ocean	0.002281429	0.00625	Max	Huber et al. (2016)	This study	10.1016/j.scitotenv.2016.03.063
Citalopram	Estuarine/Marine	Sea or Ocean	0.026	0.026	Single value	Bjorlenius et al. (2018)	This study	10.1016/j.scitotenv.2018.03.276
Clobazam	Freshwater	River/Stream	0.00582381	0.011	Max	Fick et al. (2017)	This study	10.1016/j.chemosphere.2017.02.126
Clozapine	Freshwater	River/Stream	21.025	78.3	Average	Matongo et al. (2015)[a]	This study	10.1007/s11356-015-4217-0
Clozapine	Freshwater	River/Stream	3.3325	5.59	Average	Matongo et al. (2015)[b]	This study	10.1016/j.chemosphere.2015.03.093
Clozapine	Freshwater	River/Stream	0.00284	0.0647	Max	Kondor et al. (2020)	This study	10.1016/j.envpol.2020.114893
Diazepam	Freshwater	Lake	0.005625	0.0063	Max	Pascual-Aguilar et al. (2013)	This study	10.1016/j.jhazmat.2013.07.052
Diazepam	Freshwater	Lake		0	Median	Hass et al. (2012)	der Beek (2016)	10.1002/etc.3339
Diazepam	Freshwater	River/Stream	0.00138	0.0028	Max	Fick et al. (2017)	This study	10.1016/j.chemosphere.2017.02.126

Diazepam	Freshwater	River/Stream		0.0557	Max	Mendoza et al. (2014)	This study	10.1016/j.envint.2014.05.009
Diazepam	Freshwater	River/Stream	0.00015	0.00022	Max	Kondor et al. (2020)	This study	10.1016/j.envpol.2020.114893
Diazepam	Freshwater	River/Stream	0.00031	0.00038	Max	Brieudes et al. (2017)	This study	10.1016/j.jchromb.2016.07.016
Diazepam	Freshwater	River/Stream	0.005266818	0.14	unknown	Sacher et al. 2002	der Beek (2016)	10.1002/etc.3339
Diazepam	Freshwater	River/Stream	0.0055	0.012	Average	Andreu et al. (2016)	This study	10.1016/j.scitotenv.2015.08.007
Diazepam	Freshwater	River/Stream	0.0039	0.0061	Average	Skees et al. (2018)	This study	10.1016/j.scitotenv.2018.03.060
Diazepam	Freshwater	River/Stream	0.00304	0.007	Average	Čelić et al. (2019)	This study	10.1016/j.scitotenv.2018.10.290
Diazepam	Freshwater	River/Stream	0.00023	0.00023	Average	Brieudes et al. (2016)	This study	10.1016/j.talanta.2015.06.073
Diazepam	Estuarine/Marine	Estuary	0.003936667	0.00522	Max	Fernandez-Rubio et al. (2019)	This study	10.1016/j.chemosphere.2019.02.041
Diazepam	Estuarine/Marine	Estuary		0.004	Max	Sadezky et al. (2008)	der Beek (2016)	10.1002/etc.3339
Diazepam	Estuarine/Marine	Estuary	0.00219	0.00421	Max	Zhao et al. (2015)	This study	10.1016/j.scitotenv.2015.06.055
Diazepam	Estuarine/Marine	Sea or Ocean		0.003	Max	Togola & Budzinski (2008)	der Beek (2016)	10.1002/etc.3339
Fluoxetine	Freshwater	Lake		0	Max	Ortiz (2010)	der Beek (2016)	10.1002/etc.3339
Fluoxetine	Freshwater	River/Stream	0.0018	0.0019	Max	Aminot et al. (2015)	This study	10.1007/s00216-015-9017-3
Fluoxetine	Freshwater	River/Stream	0.0718	0.1092	Average	Archer et al. (2017)	This study	10.1016/j.chemosphere.2017.01.101
Fluoxetine	Freshwater	River/Stream	0.017795	0.0289	Average	Fernandes et al. (2020)	This study	10.1016/j.chemosphere.2019.124729
Fluoxetine	Freshwater	River/Stream		0.062	Max	Park and Jeon (2021)	This study	10.1016/j.chemosphere.2020.128014
Fluoxetine	Freshwater	River/Stream	0.00435	0.0055	Average	Giebułtowicz and Nałęcz-Jawecki (2014)	This study	10.1016/j.ecoenv.2014.02.020
Fluoxetine	Freshwater	River/Stream	0.002	0.0035	Max	Ma et al. (2020)	This study	10.1016/j.envint.2020.105657

Fluoxetine	Freshwater	River/Stream		0.01	Max	Evans et al. (2017)	This study	10.1016/j.envpol.2017.06.070
Fluoxetine	Freshwater	River/Stream	0.0789	0.1441	Max	Čelić et al. (2021)	This study	10.1016/j.jhazmat.2020.124102
Fluoxetine	Freshwater	River/Stream	0.027408846	43	Max	Ortiz (2010)	der Beek (2016)	10.1002/etc.3339
Fluoxetine	Freshwater	River/Stream		0.0195	Max	Paíga et al. (2016)	This study	10.1016/j.scitotenv.2016.08.089
Fluoxetine	Freshwater	River/Stream	0.00655	0.0096	Average	Skees et al. (2018)	This study	10.1016/j.scitotenv.2018.03.060
Fluoxetine	Freshwater	River/Stream	0.0176	0.0387	Average	Čelić et al. (2019)	This study	10.1016/j.scitotenv.2018.10.290
Fluoxetine	Estuarine/Marine	Estuary	0.007763333	0.0227	Max	Fernandez-Rubio et al. (2019)	This study	10.1016/j.chemosphere.2019.02.041
Fluoxetine	Estuarine/Marine	Estuary	0.001926667	0.00197	Average	Fernandes et al. (2020)	This study	10.1016/j.chemosphere.2019.124729
Fluoxetine	Estuarine/Marine	Estuary		0.014	Max	Coelho et al. (2019)	This study	10.1016/j.jpba.2019.03.032
Fluoxetine	Estuarine/Marine	Estuary	0.00992	0.0162	Max	Reis-Santos et al. (2018)	This study	10.1016/j.marpolbul.2018.08.036
Fluoxetine	Estuarine/Marine	Estuary		0.036	Max	Anim et al. (2020)	This study	10.1016/j.marpolbul.2020.111014
Fluoxetine	Estuarine/Marine	Estuary		0.007	Max	Sadezky et al. (2008)	der Beek (2016)	10.1002/etc.3339
Fluoxetine	Estuarine/Marine	Estuary	0.074966667	0.08846	Max	Ariel et al. (2021)	This study	10.3390/ijerph18030943
Fluoxetine	Estuarine/Marine	Sea or Ocean	0.0036	0.0058	Max	Čelić et al. (2021)	This study	10.1016/j.jhazmat.2020.124102
Fluoxetine	Estuarine/Marine	Sea or Ocean		0.09	Max	Nodler et al. (2014)	This study	10.1016/j.marpolbul.2014.06.024
Fluoxetine	Estuarine/Marine	Sea or Ocean		0	Single value	Nodler et al. (2010)	der Beek (2016)	10.1002/etc.3339
Metamfetamine	Freshwater	Lake	0.0129875	0.0959	Max	Li et al. (2016)	This study	10.1016/j.envpol.2016.02.036
Metamfetamine	Freshwater	River/Stream		0.00803	Average	Cacua-Ortiz et al. (2020)	This study	10.1007/s00128-020-03028-z
Metamfetamine	Freshwater	River/Stream	0.00102	0.01074	Max	Yuan et al. (2020)	This study	10.1016/j.envpol.2020.115340

Metamfetamine	Freshwater	River/Stream		0.312	Max	Im et al. (2020)	This study	10.1016/j.scitotenv.2020.140486
Metamfetamine	Freshwater	River/Stream		0.056	Median	Lin et al. (2010)	This study	10.1016/j.chemosphere.2010.08.051
Metamfetamine	Freshwater	River/Stream		0.405	Max	Lin et al. (2010)	This study	10.1016/j.chemosphere.2010.08.051
Metamfetamine	Freshwater	River/Stream	0.025	0.025	Average	Archer et al. (2017)	This study	10.1016/j.chemosphere.2017.01.101
Metamfetamine	Freshwater	River/Stream		0.0548	Max	Mendoza et al. (2014)	This study	10.1016/j.envint.2014.05.009
Metamfetamine	Freshwater	River/Stream	0.010152381	0.0582	Average	Li et al. (2016)	This study	10.1016/j.envpol.2016.02.036
Metamfetamine	Freshwater	River/Stream	0.010975	0.0995	Max	Zhang et al. (2017)	This study	10.1016/j.scitotenv.2016.11.101
Metamfetamine	Freshwater	River/Stream	0.070865	0.2922	Max	Xu et al. (2017)	This study	10.1016/j.scitotenv.2017.05.045
Metamfetamine	Freshwater	River/Stream	0.038266667	0.0864	Average	Skees et al. (2018)	This study	10.1016/j.scitotenv.2018.03.060
Metamfetamine	Freshwater	River/Stream	0.001	0.001	Single value	van der Aa et al. (2013)	This study	10.1016/j.watres.2013.01.013
Metamfetamine	Estuarine/Marine	Estuary	0.01184375	0.0412	Max	Fernandez-Rubio et al. (2019)	This study	10.1016/j.chemosphere.2019.02.041
Mianserin	Freshwater	River/Stream	0.0039	0.009	Average	Giebułtowicz and Nałęcz-Jawecki (2014)	This study	10.1016/j.ecoenv.2014.02.020
Mianserin	Freshwater	River/Stream	0.00129	0.00206	Max	Brieudes et al. (2017)	This study	10.1016/j.jchromb.2016.07.016
Morphine	Freshwater	Lake	0.006	0.0117	Max	Pascual-Aguilar et al. (2013)	This study	10.1016/j.jhazmat.2013.07.052
Morphine	Freshwater	Lake	0.200575	0.6311	Average	Masemola et al. (2020)	This study	10.1016/j.jpba.2019.112944
Morphine	Freshwater	Lake		0	Average	Berset et al. (2010)	der Beek (2016)	10.1002/etc.3339
Morphine	Freshwater	River/Stream	0.0003	0.0003	Single value	Krizman-Matasic et al. (2018)	This study	10.1016/j.chroma.2017.12.025
Morphine	Freshwater	River/Stream		0.148	Max	Mendoza et al. (2014)	This study	10.1016/j.envint.2014.05.009
Morphine	Freshwater	River/Stream	0.00103	0.00183	Max	Brieudes et al. (2017)	This study	10.1016/j.jchromb.2016.07.016

Morphine	Freshwater	River/Stream	0.0062	0.0062	Average	Skees et al. (2018)	This study	10.1016/j.scitotenv.2018.03.060
Morphine	Freshwater	River/Stream	0.0094	0.0358	Average	Baker & Kasprzyk-Hordern (2011)	der Beek (2016)	10.1002/etc.3339
Morphine	Freshwater	River/Stream	0.007	0.007	Single value	van der Aa et al. (2013)	This study	10.1016/j.watres.2013.01.013
Morphine	Freshwater	River/Stream		0.003	Average	Andrés-Costa et al. (2016)	This study	10.1016/j.chroma.2016.07.062
Morphine	Freshwater	River/Stream		0.108	Max	Lin et al. (2010)	This study	10.1016/j.chemosphere.2010.08.051
Morphine	Estuarine/Marine	Estuary	0.033414	0.136	Max	Fernandez-Rubio et al. (2019)	This study	10.1016/j.chemosphere.2019.02.041
Oxazepam	Freshwater	Lake		0.03	Median	Hass et al. (2012)	der Beek (2016)	10.1002/etc.3339
Oxazepam	Freshwater	River/Stream	0.096	0.206	Max	Aminot et al. (2015)	This study	10.1007/s00216-015-9017-3
Oxazepam	Freshwater	River/Stream		0.0678	Max	Wang et al. (2017)	This study	10.1007/s11356-017-8922-8
Oxazepam	Freshwater	River/Stream	0.011789167	0.061	Max	Fick et al. (2017)	This study	10.1016/j.chemosphere.2017.02.126
Oxazepam	Freshwater	River/Stream	0.00265	0.00713	Max	Kondor et al. (2020)	This study	10.1016/j.envpol.2020.114893
Oxazepam	Freshwater	River/Stream	0.0261	0.051	Max	Brieudes et al. (2017)	This study	10.1016/j.jchromb.2016.07.016
Oxazepam	Freshwater	River/Stream	0.0285	0.0345	Average	Skees et al. (2018)	This study	10.1016/j.scitotenv.2018.03.060
Oxazepam	Freshwater	River/Stream	0.120308108	0.813	Single value	Togola et al. (2008)	der Beek (2016)	10.1002/etc.3339
Oxazepam	Freshwater	River/Stream	0.045276667	0.0489	Average	Brieudes et al. (2016)	This study	10.1016/j.talanta.2015.06.073
Oxazepam	Freshwater	River/Stream	0.029	0.068	Max	van der Aa et al. (2013)	This study	10.1016/j.watres.2013.01.013
Oxazepam	Freshwater	River/Stream	0.06914	0.307	Max	Camilleri et al. (2014)	This study	10.1080/03067319.2014.983494
Oxazepam	Estuarine/Marine	Estuary	0.036885	0.059	Max	Fernandez-Rubio et al. (2019)	This study	10.1016/j.chemosphere.2019.02.041
Oxazepam	Estuarine/Marine	Estuary	0.0205	0.03	Average	Aminot et al. (2016)	This study	10.1016/j.marchem.2016.05.010

Oxazepam	Estuarine/Marine	Estuary		2.183	Max	Sadezky et al. (2008)	der Beek (2016)	10.1002/etc.3339
Oxazepam	Estuarine/Marine	Sea or Ocean	0.051	0.051	Single value	Bjorlenius et al. (2018)	This study	10.1016/j.scitotenv.2018.03.276
Oxazepam	Estuarine/Marine	Sea or Ocean	0.00935	0.0127	Max	Magnér et al. (2010)	der Beek (2016)	10.1002/etc.3339
Paroxetine	Freshwater	River/Stream	0.037	0.037	Max	López et al. (2014)	This study	10.1007/s11356-014-3187-y
Paroxetine	Freshwater	River/Stream		0.0256	Max	Paíga et al. (2016)	This study	10.1016/j.scitotenv.2016.08.089
Paroxetine	Freshwater	River/Stream	0.009766667	0.0162	Average	Čelić et al. (2019)	This study	10.1016/j.scitotenv.2018.10.290
Paroxetine	Freshwater	River/Stream	0.050085556	0.225	Max	Lopez-Serna et al. (2011)	der Beek (2016)	10.1002/etc.3339
Paroxetine	Estuarine/Marine	Sea or Ocean	0.001	0.0014	Single value	Vasskog et al. 2008	der Beek (2016)	10.1002/etc.3339
Sertraline	Freshwater	River/Stream	0.0054	0.0054	Average	Fernandes et al. (2020)	This study	10.1016/j.chemosphere.2019.124729
Sertraline	Freshwater	River/Stream	0	0	Average	Giebułtowicz and Nałęcz-Jawecki (2014)	This study	10.1016/j.ecoenv.2014.02.020
Sertraline	Freshwater	River/Stream	0.011366667	0.0242	Average	Skees et al. (2018)	This study	10.1016/j.scitotenv.2018.03.060
Sertraline	Freshwater	River/Stream	0.016925	0.0304	Average	Čelić et al. (2019)	This study	10.1016/j.scitotenv.2018.10.290
Sertraline	Freshwater	River/Stream	0.0115	0.017	Average	Metcalfe et al. (2010)	der Beek (2016)	10.1002/etc.3339
Sertraline	Freshwater	River/Stream	0.07775	0.164	Max	Thomas et al. (2014)	This study	10.1111/jawr.12164
Sertraline	Estuarine/Marine	Estuary	0.0087375	0.0153	Max	Fernandez-Rubio et al. (2019)	This study	10.1016/j.chemosphere.2019.02.041
Sertraline	Estuarine/Marine	Estuary	0.202	0.304	Max	Reis-Santos et al. (2018)	This study	10.1016/j.marpolbul.2018.08.036
Sertraline	Estuarine/Marine	Estuary		0	Min	Klosterhaus et al. (2013)	der Beek (2016)	10.1002/etc.3339
Sertraline	Estuarine/Marine	Sea or Ocean		0	Single value	Nodler et al. (2010)	der Beek (2016)	10.1002/etc.3339
Temazepam	Freshwater	River/Stream		0.0132	Max	Wang et al. (2017)	This study	10.1007/s11356-017-8922-8

Temazepam	Freshwater	River/Stream	0.016223256	0.039	Max	Fick et al. (2017)	This study	10.1016/j.chemosphere.2017.02.126
Temazepam	Freshwater	River/Stream	0.00037	0.0006	Max	Kondor et al. (2020)	This study	10.1016/j.envpol.2020.114893
Temazepam	Freshwater	River/Stream	0.031	0.0609	Average	Skees et al. (2018)	This study	10.1016/j.scitotenv.2018.03.060
Temazepam	Freshwater	River/Stream	0.0278	0.0778	Max	Baker & Kasprzyk-Hordern (2013)	der Beek (2016)	10.1002/etc.3339
Temazepam	Freshwater	River/Stream	0.012	0.032	Max	van der Aa et al. (2013)	This study	10.1016/j.watres.2013.01.013
Temazepam	Estuarine/Marine	Estuary	0.02015	0.0236	Max	Fernandez-Rubio et al. (2019)	This study	10.1016/j.chemosphere.2019.02.041
Temazepam	Estuarine/Marine	Estuary		0.0378	Max	Anim et al. (2020)	This study	10.1016/j.marpolbul.2020.111014
Venlafaxine	Freshwater	Lake	0.002697143	0.0158	Single value	Li et al. (2010)	der Beek (2016)	10.1002/etc.3339
Venlafaxine	Freshwater	River/Stream	0.288	0.415	Max	López et al. (2014)	This study	10.1007/s11356-014-3187-y
Venlafaxine	Freshwater	River/Stream	0.438	0.641	Average	Fernandes et al. (2020)	This study	10.1016/j.chemosphere.2019.124729
Venlafaxine	Freshwater	River/Stream	0.05525	0.25	Average	Giebułtowicz and Nałęcz-Jawecki (2014)	This study	10.1016/j.ecoenv.2014.02.020
Venlafaxine	Freshwater	River/Stream	0.0229	0.0276	Max	Ma et al. (2020)	This study	10.1016/j.envint.2020.105657
Venlafaxine	Freshwater	River/Stream	0.014	0.0266	Max	Brieudes et al. (2017)	This study	10.1016/j.jchromb.2016.07.016
Venlafaxine	Freshwater	River/Stream		0.159	Max	Paíga et al. (2016)	This study	10.1016/j.scitotenv.2016.08.089
Venlafaxine	Freshwater	River/Stream	0.110033333	0.243	Average	Skees et al. (2018)	This study	10.1016/j.scitotenv.2018.03.060
Venlafaxine	Freshwater	River/Stream	0.16135	0.3487	Average	Čelić et al. (2019)	This study	10.1016/j.scitotenv.2018.10.290
Venlafaxine	Freshwater	River/Stream	0.016623333	0.01726	Average	Brieudes et al. (2016)	This study	10.1016/j.talanta.2015.06.073
Venlafaxine	Freshwater	River/Stream	0.117	0.295	Average	Arlos et al. (2015)	This study	10.1016/j.watres.2014.11.008
Venlafaxine	Freshwater	River/Stream	0.095797163	0.901	Average	Metcalfe et al. (2010)	der Beek (2016)	10.1002/etc.3339

Venlafaxine	Freshwater	River/Stream		0.006	Max	Park and Jeon (2021)	This study	10.1016/j.chemosphere.2020.128014
Venlafaxine	Freshwater	River/Stream		0.02	Max	Park et al. (2018)	This study	10.1016/j.scitotenv.2018.05.081
Venlafaxine	Freshwater	River/Stream	0.065	0.065	Average	Archer et al. (2017)	This study	10.1016/j.chemosphere.2017.01.101
Venlafaxine	Freshwater	River/Stream	0.053375	0.244	Max	Boix et al. (2015)	This study	10.1016/j.talanta.2014.08.005
Venlafaxine	Freshwater	River/Stream	0.3578	0.805	Max	Fonseca et al. (2020)	This study	10.1016/j.envint.2020.106004
Venlafaxine	Estuarine/Marine	Estuary	0.058578571	0.291	Max	Fernandez-Rubio et al. (2019)	This study	10.1016/j.chemosphere.2019.02.041
Venlafaxine	Estuarine/Marine	Estuary		0.015	Max	Coelho et al. (2019)	This study	10.1016/j.jpba.2019.03.032
Venlafaxine	Estuarine/Marine	Estuary	0.00231	0.0136	Max	Reis-Santos et al. (2018)	This study	10.1016/j.marpolbul.2018.08.036
Venlafaxine	Estuarine/Marine	Estuary		0.0862	Max	Anim et al. (2020)	This study	10.1016/j.marpolbul.2020.111014
Venlafaxine	Estuarine/Marine	Estuary	0.01035	0.0147	Average	Čelić et al. (2019)	This study	10.1016/j.scitotenv.2018.10.290
Venlafaxine	Estuarine/Marine	Estuary	0.01974	0.0258	Max	Ariel et al. (2021)	This study	10.3390/ijerph18030943
Venlafaxine	Estuarine/Marine	Sea or Ocean	0.003835	0.00792	Max	Huber et al. (2016)	This study	10.1016/j.scitotenv.2016.03.063
Venlafaxine	Estuarine/Marine	Sea or Ocean	0.041	0.048	Max	Bjorlenius et al. (2018)	This study	10.1016/j.scitotenv.2018.03.276
Venlafaxine	Estuarine/Marine	Sea or Ocean	0.052	0.052	Single value	Gros et al. (2012)	der Beek (2016)	10.1002/etc.3339

Table A 1.4. Data included in the principal components analysis (PCA).

ATC class	Pharmaceutical	BCF	Exposure time	pH	Temperature	Salinity
N05C	Temazepam	Q2	168.00	4.50	8.90	0.00
N05B	Oxazepam	Q1	120.00	6.87	7.00	0.00
N06A	Sertraline	Q3	168.00	6.90	20.00	0.00
N06A	Sertraline	Q4	168.00	6.90	20.00	0.00
N06A	Sertraline	Q4	168.00	6.90	20.00	0.00
N06A	Sertraline	Q4	168.00	6.90	20.00	0.00
N06A	Fluoxetine	Q1	72.00	7.00	28.00	0.00
N06A	Fluoxetine	Q1	72.00	7.00	28.00	0.00
N06A	Fluoxetine	Q1	72.00	7.00	28.00	0.00
N06A	Fluoxetine	Q1	72.00	7.00	28.00	0.00
N06A	Fluoxetine	Q1	72.00	7.00	28.00	0.00
N06A	Fluoxetine	Q1	120.00	7.00	28.00	0.00
N06A	Fluoxetine	Q1	120.00	7.00	28.00	0.00
N06A	Fluoxetine	Q1	120.00	7.00	28.00	0.00
N06A	Fluoxetine	Q1	120.00	7.00	28.00	0.00
N06A	Fluoxetine	Q2	120.00	7.00	28.00	0.00
N06B	Methylphenidate	Q1	1.00	7.00	10.03	0.00
N06B	Methylphenidate	Q1	1.00	7.00	17.07	0.00
N06B	Methylphenidate	Q1	4.00	7.00	10.03	0.00
N06B	Methylphenidate	Q1	4.00	7.00	17.07	0.00
N06B	Methylphenidate	Q1	8.00	7.00	10.03	0.00
N06B	Methylphenidate	Q1	8.00	7.00	17.07	0.00
N06B	Methylphenidate	Q1	24.00	7.00	10.03	0.00
N06B	Methylphenidate	Q1	24.00	7.00	17.07	0.00
N06B	Methylphenidate	Q1	72.00	7.00	10.03	0.00
N06B	Methylphenidate	Q1	72.00	7.00	17.07	0.00
N06B	Methylphenidate	Q1	120.00	7.00	10.03	0.00
N06B	Methylphenidate	Q1	168.00	7.00	10.03	0.00
N06A	Fluoxetine	Q4	96.00	7.10	27.00	0.00
N06A	Mianserin	Q4	96.00	7.10	27.00	0.00
N06A	Paroxetine	Q4	96.00	7.10	27.00	0.00
N06A	Sertraline	Q4	96.00	7.10	27.00	0.00
N06A	Fluoxetine	Q1	24.00	7.11	20.00	0.00
N06A	Fluoxetine	Q2	24.00	7.11	20.00	0.00
N06A	Fluoxetine	Q3	24.00	7.11	20.00	0.00
N06A	Fluoxetine	Q3	96.00	7.11	20.00	0.00
N06A	Fluoxetine	Q3	96.00	7.11	20.00	0.00
N06A	Fluoxetine	Q4	96.00	7.11	20.00	0.00
N06A	Fluoxetine	Q3	168.00	7.11	20.00	0.00
N06A	Fluoxetine	Q3	168.00	7.11	20.00	0.00

N06A	Fluoxetine	Q4	168.00	7.11	20.00	0.00
N06A	Fluoxetine	Q2	24.00	7.12	20.00	0.00
N06A	Fluoxetine	Q2	24.00	7.12	20.00	0.00
N06A	Fluoxetine	Q3	24.00	7.12	20.00	0.00
N06A	Fluoxetine	Q3	96.00	7.12	20.00	0.00
N06A	Fluoxetine	Q3	96.00	7.12	20.00	0.00
N06A	Fluoxetine	Q4	96.00	7.12	20.00	0.00
N06A	Fluoxetine	Q3	168.00	7.12	20.00	0.00
N06A	Fluoxetine	Q3	168.00	7.12	20.00	0.00
N06A	Fluoxetine	Q4	168.00	7.12	20.00	0.00
N06A	Fluoxetine	Q2	24.00	7.19	20.00	0.00
N06A	Fluoxetine	Q2	24.00	7.19	20.00	0.00
N06A	Fluoxetine	Q4	24.00	7.19	20.00	0.00
N06A	Fluoxetine	Q3	96.00	7.19	20.00	0.00
N06A	Fluoxetine	Q3	96.00	7.19	20.00	0.00
N06A	Fluoxetine	Q4	96.00	7.19	20.00	0.00
N06A	Fluoxetine	Q3	168.00	7.19	20.00	0.00
N06A	Fluoxetine	Q3	168.00	7.19	20.00	0.00
N06A	Fluoxetine	Q4	168.00	7.19	20.00	0.00
N06A	Fluoxetine	Q4	48.00	7.20	26.00	0.00
QN01A	Isoeugenol	Q2	1.00	7.29	17.10	0.00
N06A	Fluoxetine	Q4	168.00	7.40	25.00	0.00
N06A	Fluoxetine	Q3	672.00	7.40	24.37	0.00
N06A	Fluoxetine	Q3	672.00	7.40	24.37	0.00
N06A	Fluoxetine	Q3	672.00	7.40	24.37	0.00
N06A	Fluoxetine	Q4	672.00	7.40	24.37	0.00
N06A	Fluoxetine	Q4	672.00	7.40	24.37	0.00
N06A	Fluoxetine	Q4	672.00	7.40	24.37	0.00
N05B	Diazepam	Q2	168.00	7.46	18.82	0.00
N05B	Diazepam	Q2	168.00	7.46	18.82	0.00
N05B	Diazepam	Q2	168.00	7.46	18.82	0.00
N05B	Diazepam	Q2	168.00	7.46	18.82	0.00
N05B	Diazepam	Q2	168.00	7.46	18.82	0.00
N05B	Oxazepam	Q1	672.00	7.50	25.00	0.00
N05B	Oxazepam	Q1	672.00	7.50	25.00	0.00
N05B	Oxazepam	Q2	672.00	7.50	25.00	0.00
N05B	Oxazepam	Q2	672.00	7.50	25.00	0.00
N05B	Oxazepam	Q2	672.00	7.50	25.00	0.00
N05B	Oxazepam	Q2	672.00	7.50	25.00	0.00
N05B	Oxazepam	Q2	672.00	7.50	25.00	0.00
N05B	Oxazepam	Q2	672.00	7.50	25.00	0.00
N05B	Oxazepam	Q2	672.00	7.50	25.00	0.00
N05B	Oxazepam	Q2	672.00	7.50	25.00	0.00

N05B	Oxazepam	Q3	672.00	7.50	25.00	0.00
N05B	Oxazepam	Q3	672.00	7.50	25.00	0.00
N06A	Citalopram	Q1	24.00	7.50	12.00	0.00
N06A	Sertraline	Q3	96.00	7.50	22.00	0.00
N06A	Sertraline	Q3	96.00	7.50	22.00	0.00
N06A	Sertraline	Q3	96.00	7.50	22.00	0.00
N06A	Sertraline	Q3	96.00	7.50	22.00	0.00
N06A	Sertraline	Q3	96.00	7.50	22.00	0.00
N06A	Sertraline	Q4	96.00	7.50	22.00	0.00
N06A	Sertraline	Q4	96.00	7.50	22.00	0.00
N06A	Sertraline	Q4	96.00	7.50	22.00	0.00
N06A	Sertraline	Q4	96.00	7.50	22.00	0.00
N06A	Sertraline	Q4	96.00	7.50	22.00	0.00
N06A	Sertraline	Q4	96.00	7.50	22.00	0.00
N06A	Sertraline	Q4	96.00	7.50	22.00	0.00
N06A	Sertraline	Q3	168.00	7.50	22.00	0.00
N06A	Sertraline	Q3	168.00	7.50	22.00	0.00
N06A	Sertraline	Q3	168.00	7.50	22.00	0.00
N06A	Sertraline	Q3	168.00	7.50	22.00	0.00
N06A	Sertraline	Q3	168.00	7.50	22.00	0.00
N06A	Sertraline	Q4	168.00	7.50	22.00	0.00
N06A	Sertraline	Q4	168.00	7.50	22.00	0.00
N06A	Sertraline	Q4	168.00	7.50	22.00	0.00
N06A	Sertraline	Q4	168.00	7.50	22.00	0.00
N06A	Sertraline	Q4	168.00	7.50	22.00	0.00
N06A	Sertraline	Q4	168.00	7.50	22.00	0.00
N06A	Sertraline	Q4	168.00	7.50	22.00	0.00
N06A	Fluoxetine	Q2	672.00	7.50	25.00	0.00
N06A	Fluoxetine	Q2	672.00	7.50	25.00	0.00
N06A	Fluoxetine	Q2	672.00	7.50	25.00	0.00
N06A	Fluoxetine	Q2	672.00	7.50	25.00	0.00
N05A	Clozapine	Q4	672.00	7.70	20.00	0.00
N05A	Clozapine	Q4	672.00	7.70	20.00	0.00
N05A	Clozapine	Q4	672.00	7.70	20.00	0.00
N05A	Clozapine	Q4	672.00	7.70	20.00	0.00
N05A	Clozapine	Q4	672.00	7.70	20.00	0.00
N06A	Fluoxetine	Q3	24.00	7.75	26.00	0.00
N06A	Fluoxetine	Q3	24.00	7.75	26.00	0.00
N06A	Fluoxetine	Q3	24.00	7.75	26.00	0.00
N06A	Fluoxetine	Q3	34.00	7.75	26.00	0.00
N06A	Fluoxetine	Q3	34.00	7.75	26.00	0.00
N06A	Fluoxetine	Q3	34.00	7.75	26.00	0.00
N06A	Fluoxetine	Q3	34.00	7.75	26.00	0.00

N06A	Fluoxetine	Q4	34.00	7.75	26.00	0.00
N06A	Fluoxetine	Q4	34.00	7.75	26.00	0.00
N06A	Fluoxetine	Q3	48.00	7.75	26.00	0.00
N06A	Fluoxetine	Q3	48.00	7.75	26.00	0.00
N06A	Fluoxetine	Q4	48.00	7.75	26.00	0.00
N06A	Fluoxetine	Q3	72.00	7.75	26.00	0.00
N06A	Fluoxetine	Q3	72.00	7.75	26.00	0.00
N06A	Fluoxetine	Q4	72.00	7.75	26.00	0.00
N02A	Tramadol	Q1	552.00	7.80	25.00	0.00
N02A	Tramadol	Q1	552.00	7.80	25.00	0.00
N02A	Tramadol	Q1	552.00	7.80	25.00	0.00
N02A	Tramadol	Q1	552.00	7.80	25.00	0.00
N02A	Tramadol	Q1	552.00	7.80	25.00	0.00
N05C	Temazepam	Q1	720.00	7.96	23.65	0.00
N05C	Temazepam	Q2	720.00	7.96	23.65	0.00
N05C	Temazepam	Q3	720.00	7.96	23.65	0.00
N05C	Temazepam	Q4	720.00	7.96	23.65	0.00
N05B	Oxazepam	Q1	120.00	8.00	21.00	0.00
N05B	Oxazepam	Q2	120.00	8.00	21.00	0.00
N05B	Oxazepam	Q1	144.00	8.00	20.50	1.00
N05B	Oxazepam	Q2	144.00	8.00	20.50	1.00
N05B	Oxazepam	Q2	144.00	8.00	20.50	1.00
N05B	Oxazepam	Q2	144.00	8.00	20.50	1.00
N05B	Oxazepam	Q2	144.00	8.00	20.50	1.00
N05B	Oxazepam	Q2	144.00	8.00	20.50	1.00
N05B	Oxazepam	Q2	144.00	8.00	20.50	1.00
N05B	Oxazepam	Q2	144.00	8.00	20.50	1.00
N05B	Oxazepam	Q2	144.00	8.00	20.50	1.00
N05B	Oxazepam	Q2	144.00	8.00	20.50	1.00
N05B	Oxazepam	Q2	144.00	8.00	20.50	1.00
N05B	Oxazepam	Q2	144.00	8.00	20.50	1.00
N05B	Oxazepam	Q3	144.00	8.00	20.50	1.00
N05B	Oxazepam	Q3	144.00	8.00	20.50	1.00
N05B	Oxazepam	Q3	144.00	8.00	20.50	1.00
N05B	Oxazepam	Q3	144.00	8.00	20.50	1.00
N05B	Bromazepam	Q1	168.00	8.00	11.00	0.00
N05B	Bromazepam	Q1	168.00	8.00	11.00	0.00
N05B	Clobazam	Q1	168.00	8.00	11.00	0.00
N05B	Oxazepam	Q1	168.00	8.00	11.00	0.00
N05B	Oxazepam	Q1	168.00	8.00	18.00	0.00
N05B	Oxazepam	Q2	168.00	8.00	10.00	0.00
N05B	Oxazepam	Q2	168.00	8.00	10.00	0.00
N05B	Clobazam	Q2	168.00	8.00	11.00	0.00

N05B	Oxazepam	Q2	168.00	8.00	11.00	0.00
N05B	Oxazepam	Q2	168.00	8.00	18.00	0.00
N05B	Oxazepam	Q2	168.00	8.00	18.00	0.00
N05B	Oxazepam	Q2	168.00	8.00	18.00	0.00
N05C	Temazepam	Q2	168.00	8.00	11.00	0.00
N05C	Temazepam	Q2	168.00	8.00	11.00	0.00
N06A	Venlafaxine	Q3	672.00	8.00	19.00	35.00
N06A	Venlafaxine	Q4	672.00	8.00	19.00	35.00
N06A	Venlafaxine	Q4	672.00	8.00	19.00	35.00
N06A	Venlafaxine	Q4	672.00	8.00	19.00	35.00
N06A	Venlafaxine	Q2	672.00	8.05	19.00	35.10
N06A	Venlafaxine	Q3	672.00	8.05	19.00	35.10
N06A	Venlafaxine	Q4	672.00	8.05	19.00	35.10
N06A	Fluoxetine	Q4	360.00	8.10	17.30	24.80
N06A	Fluoxetine	Q4	360.00	8.10	17.30	24.80
N05C	Temazepam	Q1	720.00	8.13	20.97	0.00
N05C	Temazepam	Q1	720.00	8.13	20.97	0.00
N05C	Temazepam	Q2	720.00	8.13	20.97	0.00
N05C	Temazepam	Q2	720.00	8.13	20.97	0.00
N05C	Temazepam	Q2	720.00	8.13	20.97	0.00
N05C	Temazepam	Q3	720.00	8.13	20.97	0.00
N05C	Temazepam	Q4	720.00	8.13	20.97	0.00
N03A	Carbamazepine	Q1	168.00	8.17	12.50	0.00
N03A	Carbamazepine	Q1	168.00	8.17	12.50	0.00
N03A	Carbamazepine	Q1	168.00	8.17	12.50	0.00
N03A	Carbamazepine	Q1	168.00	8.17	12.50	0.00
N03A	Carbamazepine	Q1	168.00	8.17	12.50	0.00
N03A	Carbamazepine	Q1	168.00	8.17	12.50	0.00
N03A	Carbamazepine	Q1	168.00	8.17	12.50	0.00
N03A	Carbamazepine	Q1	336.00	8.17	12.50	0.00
N06A	Fluoxetine	Q1	168.00	8.17	12.50	0.00
N06A	Fluoxetine	Q3	168.00	8.17	12.50	0.00
N06A	Fluoxetine	Q3	168.00	8.17	12.50	0.00
N06A	Fluoxetine	Q4	168.00	8.17	12.50	0.00
N06A	Fluoxetine	Q4	168.00	8.17	12.50	0.00
N06A	Fluoxetine	Q4	168.00	8.17	12.50	0.00
N06A	Fluoxetine	Q4	336.00	8.17	12.50	0.00
N05C	Temazepam	Q1	720.00	8.20	22.37	0.00
N05C	Temazepam	Q2	720.00	8.20	22.37	0.00
N05C	Temazepam	Q3	720.00	8.20	22.37	0.00
N05C	Temazepam	Q4	720.00	8.20	22.37	0.00
N05B	Oxazepam	Q1	144.00	8.20	13.43	0.00
N05B	Oxazepam	Q1	144.00	8.20	13.43	0.00

N06A	Bupropion	Q1	504.00	8.40	22.90	0.00
N06A	Bupropion	Q1	504.00	8.40	22.90	0.00
N06A	Fluoxetine	Q2	504.00	8.40	22.90	0.00
N06A	Sertraline	Q2	504.00	8.40	22.90	0.00
N06A	Fluoxetine	Q3	504.00	8.40	22.90	0.00
N06A	Sertraline	Q3	504.00	8.40	22.90	0.00

Table A 1.5. Minimum Response Concentration (MRC) values reported for neuroactive pharmaceuticals in freshwater and brackish/marine species, concerning lethality, growth and condition, behavioural and reproductive endpoints.

Pharmaceutical	Species	Endpoint (Category)	Environment (study)	Minimum Response Concentration (MRC, µg/L)	Lower Value (Y/N)	Reference	DOI
Paroxetine	*Danio rerio*	Reproduction (fecundity and hatching)	Freshwater	10	Y	Nowakowska et al. (2020)	10.1016/j.cbpc.2019.108670
Morphine	*Danio rerio*	Lethality (LC50)	Freshwater	9915.1	Y	Ali et al. (2012)	10.1016/j.bbr.2011.11.020
Bromazepam	*Perca fluviatilis*	Behaviour (predatory avoidance and feeding)	Freshwater	8.1	Y	Cerveny et al. (2020)	10.1016/j.scitotenv.2019.134780
Clobazam	*Perca fluviatilis*	Behaviour (predatory avoidance and feeding)	Freshwater	6.9	Y	Cerveny et al. (2020)	10.1016/j.scitotenv.2019.134780
Temazepam	*Perca fluviatilis*	Behaviour (predatory avoidance and feeding)	Freshwater	9.1	Y	Cerveny et al. (2020)	10.1016/j.scitotenv.2019.134780
Metamfetamine	*Oryzias latipes*	Reproduction (fecundity and hatching)	Freshwater	0.597	Y	Liao et al. (2015)	10.1016/j.aquatox.2015.05.010
Oxazepam	*Perca fluviatilis*	Behaviour (predatory avoidance and feeding)	Freshwater	1.8	Y	Brodin et al. (2013)	10.1126/science.1226850
Clozapine	*Pimephales promelas*	Growth and Condition	Freshwater	30.8	Y	Overturf et al. (2012)	10.1007/s00244-011-9723-6
Carbamazepine	*Danio rerio*	Growth and Condition	Freshwater	1	Y	Qiang et al. (2016)	10.1007/s10646-016-1694-y
Citalopram	*Gasterosteus aculeatus*	Behaviour (predatory avoidance and feeding)	Freshwater	0.085	Y	Kellner et al. (2015)	10.1016/j.aquatox.2014.11.003
Amitriptyline	*Danio rerio*	Growth and Condition	Freshwater	0.1	Y	Yang et al. (2014)	10.1016/j.aquatox.2013.12.029
Venlafaxine	*Oncorhynchus mykiss*	Behaviour (predatory avoidance and feeding)	Freshwater	1.02	Y	Melnyk-Lamont et al. (2014)	10.1021/es504331n
Fluoxetine	*Gambusia holbrooki*	Behaviour (predatory avoidance and feeding)	Freshwater	0.008	Y	Martin et al. (2017)	10.1016/j.envpol.2016.10.010
Propofol	*Carassius auratus*	Lethality (LC50)	Freshwater	6353	Y	GholipourKanani and Ahadizadeh (2013)	10.1186/2193-1801-2-76
Bupropion	*Pimephales promelas*	Growth and Condition	Freshwater	2	Y	Painter et al. (2009)	10.1897/08-556.1

Sertraline	*Squalius cephalus*	Growth and Condition / Behaviour (predatory avoidance and feeding)	Freshwater	0.23	Y	Hubená et al. (2019)	10.7717/peerj.9356
Mianserin	*Danio rerio*	Growth and Condition	Freshwater	1.495	Y	Yang et al. (2018)	10.1016/j.ecoenv.2017.12.024
Diazepam	*Gambusia holbrooki*	Lethality (LC50)	Brackish/Marine	12700	Y	Nunes et al. (2005)	10.1016/j.ecoenv.2004.08.010
Citalopram	*Gasterosteus aculeatus*	Behaviour (predatory avoidance and feeding)	Brackish/Marine	0.15	N	Höglund et al. (2020)	10.1016/j.scitotenv.2020.140257
Fluoxetine	*Argyrosomus regius*	Growth and Condition	Brackish/Marine	2.52	N	Duarte et al. (2020)	10.1016/j.scitotenv.2020.136564
Venlafaxine	*Diplodus sargus*	Behaviour (predatory avoidance and feeding)	Brackish/Marine	100	N	Rodrigues et al. (2019)	10.1007/s10646-019-02057-7

APPENDIX 2

Supplementary material of Chapter 3

Table A 2.1. Summary of MS/MS settings used, analytes and internal standards (*) screened.

Analyte	Polarity	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)	Type
Alprazolam	+	309.0	281.0	25	103	Q
	+	309.0	205.1	39	103	q
Amitriptyline	+	278.1	233.2	15	99	Q
	+	278.1	117.2	23	99	q
* Amitriptyline-d6	+	284.1	191.1	27	99	-
Bromazepam	+	316.0	182.1	31	78	Q
	+	316.0	209.0	26	78	q
Buprenorphine	+	468.2	468.2	25	126	Q
	+	468.2	55.4	54	126	q
Bupropion	+	240.0	184.1	12	77	Q
	+	240.0	131.2	25	77	q
Caffeine	+	195.0	138.2	20	80	Q
	+	195.0	110.3	22	80	q
Carbamazepine	+	237.0	194.2	19	118	Q
	+	237.0	193.2	35	118	q
* Carbamazepine-d10	+	247.0	204.0	22	95	-
Chlorpromazine	+	319.0	86.3	19	76	Q
	+	319.0	214.0	35	76	q
Citalopram	+	325.1	109.2	27	104	Q
	+	325.1	262.1	18	104	q
Clobazam	+	301.0	259.1	19	66	Q
	+	301.0	224.0	33	66	q
Clonazepam	+	315.9	270.0	25	104	Q
	+	315.9	214.0	37	104	q
Clozapine	+	327.1	270.0	22	83	Q
	+	327.1	192.0	44	83	q
Codeine	+	300.1	215.1	23	102	Q
	+	300.1	165.2	41	102	q
* Codeine-d6	+	306.1	218.2	26	109	-
Duloxetine	+	298.1	44.3	12	74	Q
	+	298.1	123.5	50	74	q
* Flecainide-d4	+	418.9	402.2	23	108	-
Fluoxetine	+	310.1	44.3	13	84	Q

Flupentixol	+	435.1	305.1	28	116	Q
	+	435.1	265.0	34	116	q
Haloperidol	+	376.0	165.1	22	88	Q
	+	376.0	123.1	36	88	q
Hydroxyzine	+	375.1	201.1	18	97	Q
	+	375.1	166.1	35	97	q
Levomepromazine	+	329.0	100.3	20	91	Q
	+	329.0	242.1	23	91	q
Lorazepam	+	323.0	277.0	22	87	Q
	+	323.0	304.9	14	87	q
Maprotiline	+	278.1	250.2	17	99	Q
	+	278.1	219.2	24	99	q
Memantine	+	180.1	163.2	16	77	Q
	+	180.1	107.2	24	77	q
Mianserin	+	265.0	208.1	20	98	Q
	+	265.0	118.2	30	98	q
Mirtazapine	+	266.1	195.1	25	98	Q
	+	266.1	194.1	40	98	q
Oxazepam	+	287.0	241.1	22	90	Q
	+	287.0	269.1	15	90	q
* Oxazepam-d5	+	292.1	246.1	23	66	-
Paroxetine	+	330.0	192.1	20	105	Q
	+	330.0	70.4	30	105	q
Risperidone	+	411.1	191.1	27	94	Q
	+	411.1	110.2	44	94	q
* Risperidone-d4	+	415.1	195.1	27	100	-
Sertraline	+	306.0	159.0	27	100	Q
	+	306.0	275.0	12	100	q
Topiramate	-	338.1	78.0	28	103	Q
	-	338.1	96.0	21	103	q
Tramadol	+	264.1	58.4	16	82	Q
	+	264.1	246.2	10	82	q
* Tramadol-^{13}C-d3	+	268.2	58.1	18	46	-
Trihexyphenidyl	+	302.2	98.3	20	102	Q
	+	302.2	70.3	39	102	q
* Trimethoprim-^{13}C3	+	294.1	233.2	22	101	-
Venlafaxine	+	278.1	260.2	10	99	Q
	+	278.1	215.2	15	99	q
Zolpidem	+	308.1	235.2	32	103	Q
	+	308.1	263.1	24	103	q

Table A 2.2. Detailed information concerning analytes, surrogate internal standards (IS) used for quantification and limits of quantification (LOQ) for fish tissues and water samples in ng/g and ng/L, respectively.

Analyte	IS	LOQ fish (ng/g)	LOQ water (ng/L)
Alprazolam	Tramadol-^{13}C-d3	10	1
Amitriptyline	Oxazepam-d5	5	0.5
Bromazepam	Oxazepam-d5	10	10
Buprenorphine	Flecainide-d4	10	10
Bupropion	Risperidone-d4	0.1	0.01
Caffeine	Oxazepam-d5	5	5
Carbamazepine	Carbamazepine-d10	1	0.1
Chlorpromazine	Oxazepam-d5	5	1
Citalopram	Flecainide-d4	5	0.5
Clobazam	Oxazepam-d5	1	1
Clonazepam	Trimethoprim-^{13}C3	5	0.5
Clozapine	Amitriptyline-d6	1	0.1
Codeine	Codeine-d6	0.5	0.1
Duloxetine	Oxazepam-d5	1	0.1
Fluoxetine	Oxazepam-d5	5	0.5
Flupentixol	Oxazepam-d5	5	0.5
Haloperidol	Flecainide-d4	0.1	0.01
Hydroxyzine	Oxazepam-d5	0.5	0.1
Levomepromazine	Carbamazepine-d10	50	5
Lorazepam	Oxazepam-d5	5	5
Maprotiline	Oxazepam-d5	5	0.5
Memantine	Oxazepam-d5	0.5	0.1
Mianserin	Flecainide-d4	1	0.1
Mirtazapine	Tramadol-^{13}C-d3	10	1
Oxazepam	Oxazepam-d5	5	0.5
Paroxetine	Oxazepam-d5	10	1
Risperidone	Risperidone-d4	0.1	0.01
Sertraline	Oxazepam-d5	10	1
Topiramate	Amitriptyline-d6	1	0.1
Tramadol	Tramadol-^{13}C-d3	50	5
Trihexyphenidyl	Flecainide-d4	0.1	0.01
Venlafaxine	Tramadol-^{13}C-d3	0.5	0.1
Zolpidem	Tramadol-^{13}C-d3	0.5	0.1

Table A 2.3. Neuroactive pharmaceuticals in fish samples. Median (Med), Minimum (Min), and Maximum (Max) concentration values (ng/g ww) of pharmaceutical analytes detected in different fish tissues (brain, liver and muscle) collected from Douro, Tejo, Sado and Mira estuaries. The sum of concentrations (∑) and detection frequency (DF, %) per therapeutic group and for all analytes (∑ Total) are also shown. < LOQ indicates values below the Limit of Quantification (DF = 0). Therapeutic groups: OP - Opioids, AE – Antiepileptics, AP – Antipsychotics, ANX - Anxiolytics, AD - Antidepressants, PS – Psychostimulants and O – Other.

		Douro											
		D. labrax						***P. flesus***					
Therapeutic Group	**Analyte**	**Brain**	DF (%) N=5	**Liver**	DF (%) N=5	**Muscle**	DF (%) N=5	**Brain**	DF (%) N=5	**Liver**	DF (%) N=5	**Muscle**	DF (%) N=5
OP	Buprenorphine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Codeine	0.8 (0.7 - 1)	40	1.2 (0.9 - 1.5)	40	1	20	1 (0.8 - 1.7)	80	1.1 (0.6 - 1.6)	40	0.7	20
	Tramadol	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ OP		0.8 (0.7 - 1)	40	1.2 (0.9 - 1.5)	40	1	20	1 (0.8 - 1.7)	80	1.1 (0.6 - 1.6)	40	0.7	20
AE	Carbamazepine	< LOQ		< LOQ		< LOQ		1.4	20	< LOQ		< LOQ	
	Clonazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Topiramate	11 (8.2 - 46)	100	8 (4.8 - 18)	100	2.6 (1.7 – 8.1)	80	7.3 (3.6 - 14)	100	9.3 (5.3 – 17)	100	1.8 (1.6 - 2)	40
Σ AE		11 (8.2 - 46)	100	8 (4.8 - 18)	100	2.6 (1.7 – 8.1)	80	8.8 (3.6 - 14)	100	9.3 (5.3 - 17)	100	1.8 (1.6 - 2)	40
AP	Chlorpromazine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Clozapine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Flupentixol	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Haloperidol	< LOQ		< LOQ		< LOQ		< LOQ		0.2 (0.15 - 0.23)	60	< LOQ	
	Levomepromazine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Risperidone	< LOQ		0.12 (0.11 - 0.12)	40	< LOQ		0.2 (0.2 - 0.3)	40	0.1 (0.1 - 0.2)	40	0.2 (0.1 - 0.2)	40
Σ AP		< LOQ		0.12 (0.11 - 0.12)	40	< LOQ		0.2 (0.2 - 0.3)	40	0.2 (0.1 - 0.4)	80	0.2 (0.1 - 0.2)	40
ANX	Alprazolam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Bromazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Clobazam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Hydroxyzine	< LOQ		< LOQ		< LOQ		< LOQ		1.4	20	< LOQ	
	Lorazepam	< LOQ		< LOQ		< LOQ		< LOQ		5.3	20	< LOQ	

	Oxazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ ANX		< LOQ		< LOQ		< LOQ		< LOQ		6.7	20	< LOQ	
AD	Amitriptyline	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Bupropion	< LOQ		< LOQ		< LOQ		< LOQ		0.2 (0.1 - 0.4)	60	< LOQ	
	Citalopram	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Duloxetine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Fluoxetine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Maprotiline	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Mianserin	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Mirtazapine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Paroxetine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Sertraline	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Venlafaxine	0.7	20	< LOQ		2.1	20	1.1	20	2 (1.8 - 2.3)	40	0.5	20
Σ AD		0.7	20	< LOQ		2.1	20	1.1	20	1.9 (0.2 - 2.6)	60	0.5	20
PS	Caffeine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		5.3	20
Σ PS		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		5.3	20
O	Memantine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Trihexyphenidyl	< LOQ		< LOQ		< LOQ		0.1	20	< LOQ		< LOQ	
	Zolpidem	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ O		< LOQ		< LOQ		< LOQ		0.1	20	< LOQ		< LOQ	
Σ Total		11 (8.2 - 46)	100	9.6 (4.8 - 18)	100	3.1 (1 - 8.1)	100	11 (3.6 - 17)	100	11 (5.7 - 27)	100	1.6 (0.1 - 6.1)	100

		Mira											
		D. labrax						*D. sargus*					
Therapeutic Group	**Analyte**	**Brain**	DF (%) N=2	**Liver**	DF (%) N=2	**Muscle**	DF (%) N=2	**Brain**	DF (%) N=2	**Liver**	DF (%) N=2	**Muscle**	DF (%) N=2
OP	Buprenorphine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Codeine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Tramadol	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ OP		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
AE	Carbamazepine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Clonazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Topiramate	19 (15 - 22)	100	13 (11 - 14)	100	3.3 (3 - 3.6)	100	< LOQ		< LOQ		< LOQ	
Σ AE		19 (15 - 22)	100	13 (11 - 14)	100	3.3 (3 - 3.6)	100	< LOQ		< LOQ		< LOQ	
AP	Chlorpromazine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Clozapine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Flupentixol	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Haloperidol	< LOQ		0.2 (0.1 - 0.2)	100	< LOQ		< LOQ		< LOQ		< LOQ	
	Levomepromazine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Risperidone	0.2 (0.2 - 0.3)	100	0.29 (0.27 - 0.3)	100	0.1 (0.1 - 0.2)	100	0.2 (0.1 - 0.3)	100	0.18 (0.16 - 0.2)	100	0.1 (0.1 - 0.2)	100
Σ AP		0.2 (0.2 - 0.3)	100	0.5 (0.4 - 0.5)	100	0.1 (0.1 - 0.2)	100	0.2 (0.1 - 0.3)	100	0.18 (0.16 - 0.2)	100	0.1 (0.1 - 0.2)	100
ANX	Alprazolam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Bromazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Clobazam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Hydroxyzine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Lorazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Oxazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ ANX		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
AD	Amitriptyline	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	

	Bupropion	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Citalopram	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Duloxetine	< LOQ		1.7	50	< LOQ		< LOQ		< LOQ		< LOQ	
	Fluoxetine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Maprotiline	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Mianserin	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Mirtazapine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Paroxetine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Sertraline	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Venlafaxine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ AD		< LOQ		1.7	50	< LOQ		< LOQ		< LOQ		< LOQ	
PS	Caffeine	7.5	50	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ PS		7.5	50	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
O	Memantine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Trihexyphenidyl	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Zolpidem	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ O		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ Total		22 (22 - 23)	100	14 (13 - 15)	100	3.4 (3.1 - 3.7)	100	0.2 (0.1 - 0.3)	100	0.18 (0.16 - 0.2)	100	0.1 (0.1 - 0.2)	100

		Mira											
		H. didactylus						*S. aurata*					
Therapeutic Group	**Analyte**	**Brain**	DF (%) N=3	**Liver**	DF (%) N=3	**Muscle**	DF (%) N=3	**Brain**	DF (%) N=4	**Liver**	DF (%) N=4	**Muscle**	DF (%) N=4
OP	Buprenorphine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Codeine	1.3 (1.2 - 1.4)	67	1.2	33	< LOQ		< LOQ		0.8	25	1.1	25
	Tramadol	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ OP		1.3 (1.2 - 1.4)	67	1.2	33	< LOQ		< LOQ		0.8	25	1.1	25
AE	Carbamazepine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Clonazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Topiramate	< LOQ		1.6	33	< LOQ		5.1 (3 - 10)	75	4.8 (1.9 - 9.7)	75	1.6	25
Σ AE		< LOQ		1.6	33	< LOQ		5.1 (3 - 10)	75	4.8 (1.9 - 9.7)	75	1.6	25
AP	Chlorpromazine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Clozapine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Flupentixol	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Haloperidol	< LOQ		< LOQ		< LOQ		< LOQ		0.14	25	< LOQ	
	Levomepromazine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Risperidone	0.2 (0.1 - 0.2)	100	0.12 (0.11 - 0.13)	67	0.2 (0.1 - 0.3)	67	0.2 (0.1 - 0.2)	100	0.2 (0.1 - 0.6)	100	0.2 (0.1 - 0.2)	100
Σ AP		0.2 (0.1 - 0.2)	100	0.12 (0.11 - 0.13)	67	0.2 (0.1 - 0.3)	67	0.2 (0.1 - 0.2)	100	0.3 (0.1 - 0.6)	100	0.2 (0.1 - 0.2)	100
ANX	Alprazolam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Bromazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Clobazam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Hydroxyzine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Lorazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Oxazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ ANX		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
AD	Amitriptyline	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	

	Bupropion	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Citalopram	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Duloxetine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Fluoxetine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Maprotiline	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Mianserin	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Mirtazapine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Paroxetine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Sertraline	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Venlafaxine	< LOQ		< LOQ		1	33	< LOQ		< LOQ		< LOQ	
Σ AD		< LOQ		< LOQ		1	33	< LOQ		< LOQ		< LOQ	
PS	Caffeine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ PS		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
O	Memantine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Trihexyphenidyl	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Zolpidem	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ O		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ Total		1.4 (0.1 - 1.6)	100	1.5 (0.1 - 2.9)	67	0.7 (0.3 - 1.1)	67	4.2 (0.1 - 10)	100	4 (0.2 - 10)	100	0.7 (0.2 - 1.7)	100

		Sado											
		D. bellottii						*D. labrax*					
Therapeutic Group	**Analyte**	**Brain**	DF (%) N=5	**Liver**	DF (%) N=6	**Muscle**	DF (%) N=6	**Brain**	DF (%) N=1	**Liver**	DF (%) N=1	**Muscle**	DF (%) N=1
OP	Buprenorphine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Codeine	0.6	20	0.9	17	0.7	17	< LOQ		0.8	100	< LOQ	
	Tramadol	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ OP		0.6	20	0.9	17	0.7	17	< LOQ		0.8	100	< LOQ	
AE	Carbamazepine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Clonazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Topiramate	< LOQ		< LOQ		< LOQ		2.6	100	3.7	100	< LOQ	
Σ AE		< LOQ		< LOQ		< LOQ		2.6	100	3.7	100	< LOQ	
AP	Chlorpromazine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Clozapine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Flupentixol	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Haloperidol	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Levomepromazine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Risperidone	0.2 (0.1 - 0.2)	100	0.2 (0.1 - 0.3)	67	0.2 (0.1 - 0.2)	83	0.2	100	0.2	100	0.2	100
Σ AP		0.2 (0.1 - 0.2)	100	0.2 (0.1 - 0.3)	67	0.2 (0.1 - 0.2)	83	0.2	100	0.2	100	0.2	100
ANX	Alprazolam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Bromazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Clobazam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Hydroxyzine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Lorazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Oxazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ ANX		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
AD	Amitriptyline	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	

	Bupropion	< LOQ		< LOQ		0.1	17	< LOQ		< LOQ		< LOQ	
	Citalopram	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Duloxetine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Fluoxetine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Maprotiline	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Mianserin	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Mirtazapine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Paroxetine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Sertraline	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Venlafaxine	0.7 (0.5 - 0.8)	40	1	17	0.6 (0.5 - 0.6)	33	0.6	100	< LOQ		< LOQ	
Σ AD		0.7 (0.5 - 0.8)	40	1	17	0.5 (0.1 - 0.6)	50	0.6	100	< LOQ		< LOQ	
PS	Caffeine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ PS		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
O	Memantine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Trihexyphenidyl	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Zolpidem	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ O		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ Total		0.2 (0.1 - 1.3)	100	0.3 (0.2 - 1)	83	0.2 (0.1 - 1.5)	83	3.3	100	4.6	100	0.2	100

		Sado											
		H. didactylus						*S. aurata*					
Therapeutic Group	**Analyte**	**Brain**	DF (%) N = 5	**Liver**	DF (%) N = 6	**Muscle**	DF (%) N = 6	**Brain**	DF (%) N = 1	**Liver**	DF (%) N = 1	**Muscle**	DF (%) N = 1
OP	Buprenorphine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Codeine	0.5	20	1.3	17	< LOQ		0.8	100	< LOQ		< LOQ	
	Tramadol	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ OP		0.5	20	1.3	17	< LOQ		0.8	100	< LOQ		< LOQ	
AE	Carbamazepine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Clonazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Topiramate	1.5 (1.3 - 12)	60	6.4	17	< LOQ		< LOQ		< LOQ		< LOQ	
Σ AE		1.5 (1.3 - 12)	60	6.4	17	< LOQ		< LOQ		< LOQ		< LOQ	
AP	Chlorpromazine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Clozapine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Flupentixol	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Haloperidol	< LOQ		0.1	17	< LOQ		< LOQ		< LOQ		< LOQ	
	Levomepromazine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Risperidone	0.2 (0.2 - 0.3)	60	0.1 (0.1 - 0.2)	83	0.11 (0.11 - 0.14)	50	0.1	100	0.2	100	0.1	100
Σ AP		0.2 (0.2 - 0.3)	60	0.1 (0.1 - 0.3)	83	0.11 (0.11 - 0.14)	50	0.1	100	0.2	100	0.1	100
ANX	Alprazolam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Bromazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Clobazam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Hydroxyzine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Lorazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Oxazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ ANX		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
AD	Amitriptyline	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Bupropion	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	

	Citalopram	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Duloxetine	1.6	20	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Fluoxetine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Maprotiline	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Mianserin	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Mirtazapine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Paroxetine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Sertraline	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Venlafaxine	1	20	2.7 (1.1 - 4.3)	33	1.8 (1.1 - 2.5)	33	< LOQ		< LOQ		< LOQ	
Σ AD		2.7	20	2.7 (1.1 - 4.3)	33	1.8 (1.1 - 2.5)	33	< LOQ		< LOQ		< LOQ	
PS	Caffeine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ PS		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
O	Memantine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Trihexyphenidyl	< LOQ		0.1	17	< LOQ		< LOQ		< LOQ		< LOQ	
	Zolpidem	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ O		< LOQ		0.1	17	< LOQ		< LOQ		< LOQ		< LOQ	
Σ Total		2.2 (0.2 - 13)	100	2.9 (0.1 - 6.6)	83	0.1 (0.1 - 2.5)	83	0.9	100	0.2	100	0.1	100

		Sado						Tejo					
		S. solea						*D. labrax*					
Therapeutic Group	**Analyte**	**Brain**	DF (%) N=2	**Liver**	DF (%) N=2	**Muscle**	DF (%) N=2	**Brain**	DF (%) N=6	**Liver**	DF (%) N=7	**Muscle**	DF (%) N=7
OP	Buprenorphine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Codeine	< LOQ		< LOQ		< LOQ		0.6 (0.6 - 0.7)	33	0.7	14	1	14
	Tramadol	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ OP		< LOQ		< LOQ		< LOQ		0.6 (0.6 - 0.7)	33	0.7	14	1	14
AE	Carbamazepine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Clonazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Topiramate	13 (3.5 - 22)	100	7.1 (4.6 - 9.6)	100	< LOQ		15 (8.7 - 207)	100	16 (3.8 - 67)	86	2.3 (1.5 - 12)	100
Σ AE		13 (3.5 - 22)	100	7.1 (4.6 - 9.6)	100	< LOQ		15 (8.7 - 207)	100	16 (3.8 - 67)	86	2.3 (1.5 - 12)	100
AP	Chlorpromazine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Clozapine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Flupentixol	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Haloperidol	< LOQ		0.2	50	< LOQ		< LOQ		< LOQ		< LOQ	
	Levomepromazine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Risperidone	0.29 (0.26 - 0.31)	100	0.2 (0.2 - 0.3)	100	0.2 (0.2 - 0.3)	100	0.2 (0.1 - 0.3)	83	0.1 (0.1 - 0.2)	57	0.1 (0.1 - 0.4)	100
Σ AP		0.29 (0.26 - 0.31)	100	0.3 (0.2 - 0.5)	100	0.2 (0.2 - 0.3)	100	0.2 (0.1 - 0.3)	83	0.1 (0.1 - 0.2)	57	0.1 (0.1 - 0.4)	100
ANX	Alprazolam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Bromazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Clobazam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Hydroxyzine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Lorazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Oxazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ ANX		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
AD	Amitriptyline	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	

	Bupropion	< LOQ		0.1	50	< LOQ		< LOQ		< LOQ		< LOQ	
	Citalopram	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Duloxetine	< LOQ		< LOQ		< LOQ		< LOQ		3	14	< LOQ	
	Fluoxetine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Maprotiline	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Mianserin	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Mirtazapine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Paroxetine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Sertraline	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Venlafaxine	< LOQ		< LOQ		< LOQ		0.6 (0.5 - 1.5)	50	1.5 (1.3 - 1.7)	29	0.8	14
Σ AD		< LOQ		0.1	50	< LOQ		0.6 (0.5 - 1.5)	50	3 (1.7 - 4.3)	29	0.8	14
PS	Caffeine	9.7	50	12	50	< LOQ		5.5	17	< LOQ		< LOQ	
Σ PS		9.7	50	12	50	< LOQ		5.5	17	< LOQ		< LOQ	
O	Memantine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Trihexyphenidyl	< LOQ		0.1	50	< LOQ		< LOQ		< LOQ		< LOQ	
	Zolpidem	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ O		< LOQ		0.1	50	< LOQ		< LOQ		< LOQ		< LOQ	
Σ Total		18 (13 – 23)	100	13 (9.8 - 17)	100	0.2 (0.2 - 0.3)	100	19 (9.5 - 207)	100	13 (3.8 - 69)	100	2.5 (1.6 - 13)	100

		Tejo											
		H. didactylus						*S. solea*					
Therapeutic Group	**Analyte**	**Brain**	DF (%) N=5	**Liver**	DF (%) N=5	**Muscle**	DF (%) N=5	**Brain**	DF (%) N=4	**Liver**	DF (%) N=3/6 *	**Muscle**	DF (%) N=6
OP	Buprenorphine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Codeine	0.9	20	< LOQ		< LOQ		< LOQ		0.9	33	0.7 (0.7 - 0.7)	33
	Tramadol	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ OP		0.9	20	< LOQ		< LOQ		< LOQ		0.9	33	0.7 (0.7 - 0.7)	33
AE	Carbamazepine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Clonazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Topiramate	5.5 (2.3 - 12)	100	1.7 (1.1 - 5)	60	1.3 (1.2 - 1.5)	80	30 (12 - 167)	100	14 (6 - 86)	83*	3.5 (1.5 - 20)	100
Σ AE		5.5 (2.3 - 12)	100	1.7 (1.1 – 5)	60	1.3 (1.2 - 1.5)	80	30 (12 - 167)	100	14 (6 - 86)	83	3.5 (1.5 – 20)	100
AP	Chlorpromazine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Clozapine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	*	< LOQ	
	Flupentixol	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Haloperidol	< LOQ		0.3 (0.1 - 0.5)	80	< LOQ		< LOQ		< LOQ		< LOQ	
	Levomepromazine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Risperidone	0.12 (0.1 - 0.12)	60	0.2 (0.1 - 0.3)	60	0.16 (0.15 - 0.17)	40	0.2 (0.2 - 0.3)	75	0.2 (0.2 - 0.2)	67	0.1 (0.1 - 0.2)	50
Σ AP		0.12 (0.1 - 0.12)	60	0.4 (0.3 - 0.5)	100	0.16 (0.15 - 0.17)	40	0.2 (0.2 - 0.3)	75	0.2 (0.2 - 0.2)	67	0.1 (0.1 - 0.2)	50
ANX	Alprazolam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Bromazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	*	< LOQ	
	Clobazam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	*	< LOQ	
	Hydroxyzine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Lorazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	*	< LOQ	
	Oxazepam	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ ANX		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	

AD	Amitriptyline	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Bupropion	< LOQ		< LOQ		< LOQ		< LOQ		0.3	33	< LOQ	
	Citalopram	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Duloxetine	< LOQ		< LOQ		< LOQ		< LOQ		1.7	33	< LOQ	
	Fluoxetine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Maprotiline	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Mianserin	< LOQ		2	20	1.1	20	< LOQ		< LOQ		< LOQ	
	Mirtazapine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Paroxetine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Sertraline	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Venlafaxine	3.3 (1.4 - 6.5)	80	2.3 (0.6 - 4.1)	40	1.7 (1.2 - 2.1)	40	8 (3.3 - 13)	50	2.5	33	0.68 (0.66 - 0.7)	33
Σ AD		3.3 (1.4 - 6.5)	80	3.4 (0.6 - 6.1)	40	2.2 (2.1 - 2.3)	40	8 (3.3 - 13)	50	2.2 (1.7 - 2.8)	67	0.68 (0.66 - 0.7)	33
PS	Caffeine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ PS		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
O	Memantine	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
	Trihexyphenidyl	0.1	20	< LOQ		0.2	20	< LOQ		< LOQ		< LOQ	
	Zolpidem	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ		< LOQ	
Σ O		0.1	20	< LOQ		0.2	20	< LOQ		< LOQ		< LOQ	
Σ Total		8.5 (3.7 - 16)	100	2.1 (0.3 - 6.6)	100	1.5 (1.4 - 3.5)	100	30 (25 - 171)	100	12 (3 - 86)	100	4.2 (1.6 - 21)	100

Figure A 2.1. Principal component analysis (PCA) of the summed concentrations of neuroactive pharmaceuticals per therapeutic group, detected in water samples collected from the four estuarine systems, Douro (□, N=3), Tejo (∇, N=9), Sado (Δ, N=7) and Mira (◊, N=6). Therapeutic groups are the following: PS - Psychostimulants, OP - Opioids, AD - Antidepressants, ANX - Anxiolytics, AE - Antiepileptics, AP - Antipsychotics and O - Other.

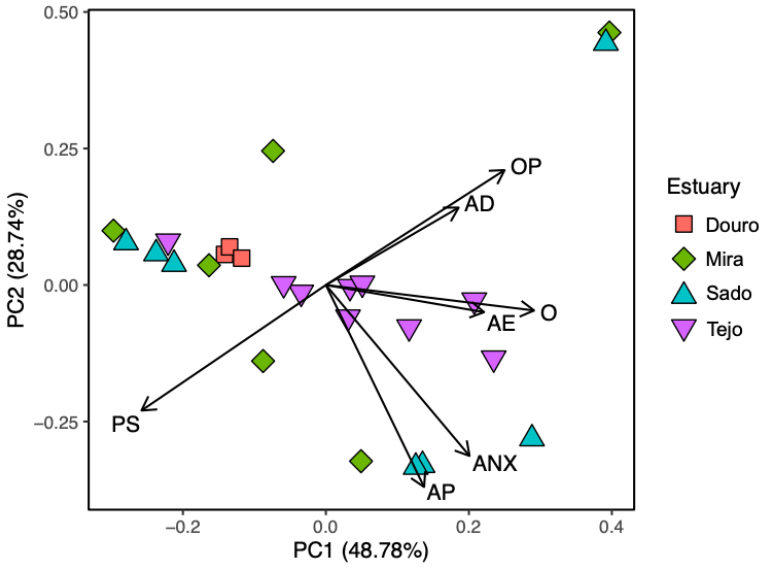
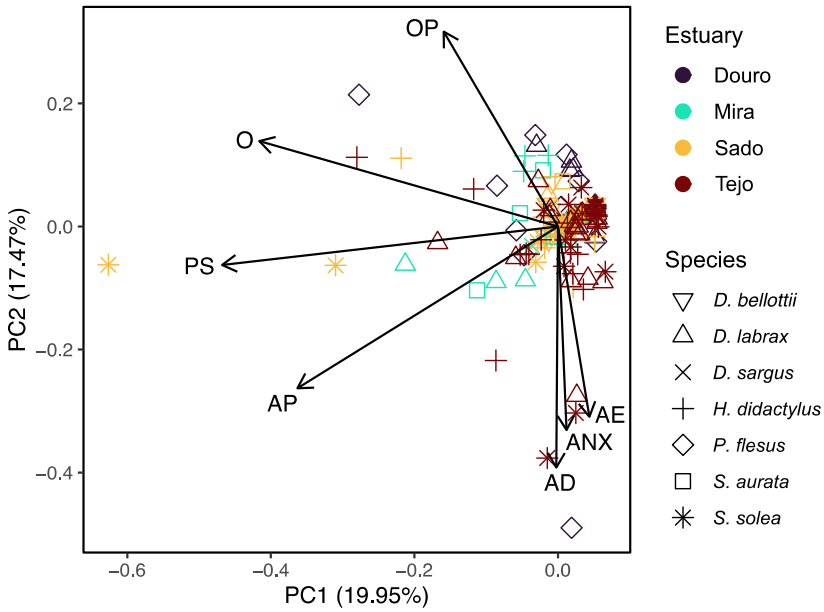


Figure A 2.2. Principal component analysis (PCA) of the summed concentrations of neuroactive pharmaceuticals per therapeutic group, detected in fish from all 7 species (*D. bellottii*, *D. labrax*, *D. sargus*, *H. didactylus*, *P. flesus*, *S. aurata*, *S. solea*) collected from the four estuarine systems, Douro, Tejo, Sado and Mira. Therapeutic groups are the following: PS - Psychostimulants, OP - Opioids, AD - Antidepressants, ANX - Anxiolytics, AE - Antiepileptics, AP - Antipsychotics and O - Other.



APPENDIX 3

Supplementary material of Chapter 5

Analytical procedures and methodologies for detection and quantification of pharmaceutical contaminants in water and fish muscle tissues via UHPLC-TOF-MS

Water sample extraction, purification and concentration was adapted from Pereira et al. (2015) and Sousa et al. (2011). Five hundred mL of each water sample were filtered through glass microfiber filter (1.2µm), cellulose nitrate filter (0.45µm) and Sartolon Polyamid filter (0.2µm). Samples were then purified with OASIS HLB cartridges (no need for pre-conditioning) and cartridges washed with 5 mL of methanol:water (10:90) and allowed to dry for 15 minutes at low vacuum pressure. Elution was performed with 6 mL of methanol and the extract dried under N_2 flow in a bath at 40ºC. The dry residue was dissolved with 500 µL of methanol:water (3:97), filtered through a PVDF Mini-uniprep TM filter (0.45 µm), and transferred to vials ready for subsequent injection and quantification by Ultra-high performance liquid chromatography and time-of-flight mass spectrometry (UHPLC-TOF-MS).

Tissue sample extraction, purification and concentration was performed as an extension of the method from Freitas et al. (2014). Shortly, sample tissues (2g) were weighted and extracted with 5mL of acetonitrile and 1mL of 0.1M EDTA. After homogenization and centrifugation, the supernatant was evaporated to nearly dryness (until 0.5 mL) under a gentle stream of nitrogen at 40°C. Five hundred µL of 0.1% formic acid were added to the residue, filtered through a PVDF mini-uniprep™ and injected into the UHPLC-TOF-MS for detection and quantification.

Chromatographic separation and mass spectrometry detection were performed with an UHPLC Nexera X2 Shimadzu coupled with a Triple TOFTM 5600+ from AB Sciex (UHPLC-TOF-MS). Methodology was based on Chau et al., 2017; Freitas et al., 2014; Leston et al., 2016; Santos et al 2016. The electrospray ion source was used in positive (ESI+) mode with full-scan data acquisition from 100 to 920 Da. Identification and quantification of target compounds were performed with the PeakViewTM and MultiQuantTM software. Identification was based on the exact mass with an error below 10 ppm and variation of retention time to a maximum of 2.5 % and the isotope ratio difference lower than 10%. The TOF-MS detector was calibrated between each 10 injections to guarantee the accurate mass resolution. The UHPLC system consisted of a vacuum degasser, an autosampler and a binary pump equipped with an

analytical reverse-phase column Zorbax Eclipse Plus C18 - 2.1X50mm, 1.8 micron (Agilent). The flow rate was 0.5 mL min^{-1} with the following mobile phases: [A] formic acid 0.1% (v/v) in water and [B] methanol. The gradient program used was: 0-5 min from 97% to 40% [A]; 5-9 min from 40% to 0% [A]; 9-10 min from 0% back to 97% [A]; 11-12 min 97% [A]. The column was maintained at 40°C, and the autosampler at 10°C with an injection volume of 10 µL. For extraction and purification all reagents and solvents were of analytical grade and for the mobile phase they were of high-performance liquid chromatography grade. Methanol, acetonitrile and formic acid were supplied by Merck (Darmstadt, Germany), whereas all standards were supplied by Sigma-Aldrich (Madrid, Spain). Stock solutions (1 mg mL^{-1}) of each standard were prepared in methanol, except for beta-lactams that were prepared in water, and subsequently diluted to obtain convenient concentrations for the quantification of target compounds in the extracts. All standard solutions were stored away from light at -20ºC. The optimized methods were validated as a quantitative method by evaluating the following parameters: specificity, selectivity, precision, linearity, limit of detection (LOD) and limit of quantification (LOQ). Limits of detection and quantification (LOQ), % of repeatability, reproducibility and recovery are summarised in Table A1.

Table A 3.1. Summary of limits of detection (LOD), quantification (LOQ), repeatability, reproducibility and recovery (%) of pharmaceutical analysis in fish muscle tissues and water samples.

	Matrix	**LOD (µg/kg)**	**LOQ (µg/kg)**	**Repeatability (%)**	**Reproducibility (%)**	**Recovery (%)**
Fluoxetine	Muscle	0.86	2.86	7.7	11.5	89.7
	Water	0.01	0.03	5.2	7.8	109.3
Diclofenac	Muscle	1.08	5.77	13.14	23.27	121.52
	Water	0.02	0.05	5.9	8.8	106.0
Propranolol	Muscle	1.51	5.05	6.1	9.1	91.8
	Water	0.06	0.21	4.5	6.5	100.9

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Detailed protocols for biomarkers quantification

For biomarkers quantification different fish tissues were dissected, namely liver, brain, muscle, and heart. Tissue samples were homogenized in cold 100 mM monobasic potassium phosphate/dibasic potassium phosphate (K_2HPO_4/KH_2PO_4) buffer (pH 7.4) containing 0.15 M KCl (potassium chloride), 0.1 mM PMSF (phenylmethylsulfonyl fluoride), 1 mM DTT (dithiothreitol) and 1 mM EDTA (ethylenediaminetetraacetic acid) to avoid protein degradation. Tissues were homogenized with tissue:buffer ratios between 1:5 and 1:10 (w/v).

Liver homogenates were aliquoted for DNA damage (DNAd), lipid peroxidation (LPO) quantification. Butylated hydroxytoluene (BHT) at 4% in methanol was added (1:15 v/v sample) to the LPO aliquots to prevent further lipid peroxidation The remaining liver homogenate was centrifuged at 12000 g for 20 min at 4°C, and aliquoted for superoxide dismutase (SOD), catalase (CAT), ethoxyresorufin-*O*-deethylase (EROD) and glutathione *S*-transferase (GST) determination.

Muscle homogenates were first aliquoted for the determination of LPO, DNAd, total carbohydrates (CBH), proteins (PT) and lipids (LP) content. The remaining homogenate was further centrifuged at 1000 g for 10 min at 4ºC for the determination of electron transport system (ETS) activity, and at 3000 g for 5 min at 4ºC for lactate dehydrogenase (LDH) and isocitrate dehydrogenase (IDH) enzymes activities.

Heart homogenates were used for ETS, LDH and IDH activity measurements. Heart homogenates were centrifuged at 1000 g for 10 min at 4ºC for ETS determination and further at 3000 g for 5 min at 4ºC for LDH and IDH activity measurements.

Brain homogenates were used for the measurement of acetylcholinesterase (AChE) activity, after centrifugation at 11000 g for 3 min at 4°C.

All biomarker responses were determined using a Sinergy HT Microplate Reader (Bio-Tek Instruments, Vermont, USA) and each reading was done in triplicate, using buffer as blank reaction.

Protein content was quantified following Bradford's method: Bradford solution is added to each replicate of sample and incubated for a 15 min period (at room temperature and light protected) after which absorbance is measured at 595 nm. Bovine serum albumin solution (1 mg mL^{-1}) was used as protein standard.

Superoxide dismutase (SOD) activity was measured according to Mccord and Fridovich, 1969), with slight modifications. Briefly, 140 µL of 50 mM phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 1.5 mM hypoxanthine and 0.15 mM cytochrome c, as well as 60 µL of 130 mU mL^{-1} xanthine oxidase were added to the sample (10 µL). The reduction of cytochrome c by the xanthine oxidase/hypoxanthine system was measured at a wavelength of 550 nm. One unit of SOD is the amount of enzyme that inhibits the reduction of cytochrome c by 50%. SOD activity was expressed as U mg^{-1} of total protein concentration.

Catalase (CAT) activity in liver tissues was determined according to Aebi (1974), following substrate consumption, as a decrease in absorbance at 240 nm. Briefly, 130 µL of 50 mM phosphate buffer were added to 20 µL of sample, and the reaction was started with the addition of 150 µL of substrate (30 mM H_2O_2 in 50 mM phosphate buffer, pH 7). CAT activity was then calculated as the difference in absorbance per unit of time ($\varepsilon = -0.04$ mM^{-1} cm^{-1}) and expressed as µmol per minute per mg of total protein concentration.

Ethoxyresorufin-O-deethylase (EROD) activity was determined following Burke and Mayer (1974) method, with few adaptations by Fernandes et al. (2002). The reaction was initiated with the addition of 10 µL of NADPH (8.33 mg mL^{-1}) to 190 µL of 7-ethoxyresorufin solution (0.1 mg mL^{-1} in 100 mM phosphate buffer, pH 7.4) and 100 µL of sample, at 30 °C

for 20 min. Fluorescence from 7-hydroxyresorufin was measured at 537/583 nm excitation/emission wavelengths, and resorufin sodium salt was used as standard. Activity was calculated as the amount of resorufin (ρmol) generated per mg of total protein per minute of reaction time.

Glutathione S-transferase (GST) activity was determined following Habig et al. (1974). Briefly, the conjugation of glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) by GST was measured through changes in absorbance at 340 nm ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$), for 3 min. The assay started with the addition of 250 µL of a final reaction mixture containing 100 mM phosphate buffer (pH 6.5), 20 mM CDNB and 20 mM reduced glutathione, to 50 µL of sample. GST activity was expressed as nmol CDNB conjugate formed per mg of total protein per minute of reaction.

Lipid peroxidation (LPO) was determined according to Ohkawa et al. (1979). The reaction of thiobarbituric acid reactive substances (TBARS) with 2-thiobarbituric acid (TBA) occurred after incubation of 500 µL of TCA 12%, 450 µL of 60 mM Tris-HCl (pH 7.4) containing 0.1 mM EDTA and 500 µL of TBA 0.73% with 50 µL of sample for 60 min, at 97 °C. Samples were cooled on ice and centrifuged at 13400g for 3 min, and absorbance was measured at 535 nm ($\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$). LPO was expressed as nmol of TBARS formed per mg of wet weight.

DNA damage (DNAd) was determined following the DNA alkaline precipitation assay by Olive (1988). Samples (50 µL) were first mixed with 250 µL of a 2% SDS solution containing 10 mM EDTA, 10 mM Trisbase (pH 12.4) and 50 mM NaOH. Then, 0.12 M KCl solution (250 µL) was added and the mixture was incubated at 60 °C for 10 min. After cooling down on ice for 15 min, it was centrifuged at 8000g for 5 min, at 4 °C. Following the addition of 200 µL of Hoechst dye (1 µg mL^{-1} in 0.1 M K-phosphate buffer, pH 7.4) to 50 µL of the mixture, DNA concentration in the supernatant was determined at 360 nm/460 nm of excitation/emission wavelengths. Fluorescence values were compared to a DNA standard curve and DNAd was expressed as µg DNA per mg of wet weight.

Acetylcholinesterase (AChE) was determined according to Ellman et al. (1961), adapted to microplate (Guilhermino et al., 1996). Briefly, 250 µL of a reaction mixture containing 100 mM phosphate buffer (pH 7.2), 75 mM acetylthiocholine and 10 mM DTNB (5,5'-dithio-bis(2-nitrobenzoic acid)) were added to 50 µL of sample. The reaction of thiocholine with DTNB to produce the yellow anion TNB, was followed at 412 nm ($\varepsilon = 13.6\ mM^{-1}cm^{-1}$), every 20 secs for 10 min, and the enzymatic activity was expressed in nmol of substrate hydrolyzed per minute per mg of total protein.

LDH activity determination was assessed using the methods described by Vassault, (1983) and Diamantino et al., (2001), by measuring the efficiency of this enzyme to convert pyruvate to lactate in the presence of NADH, which results in NADH oxidation and consequent decrease in absorbance. To perform the reaction, 200 µL of NADH (0.27 mM) were added to samples (50 µL), followed by 50 µL of sodium pyruvate (7.08 mM). The absorbance resulting from the reaction kinetics was read at 340 nm, every 10 seconds, for 5 min. Results were expressed as nmol min^{-1} mg $protein^{-1}$, using a molar extinction coefficient of 6.22×10^3 $M.cm^{-1}$.

The activity of IDH was determined by following the increase in NADPH along with the decarboxylation of isocitrate by IDH, following Ellis and Goldberg, (1971) method, adapted by Lima et al., (2007). In this reaction, 50 µL of sample were added to 200 µL of reaction buffer containing 2 mM Mangan(II)-chlorid-tetrahyhot (15mL), 50 mM Tris(hydroxymethyl)-aminomethan (40mL at pH 7.8) and 7 mM DL-isocitric acid (15 mL), to which 50 µL of $NADP^+$ solution (0.5 mM) were added right away. The change in absorbance was measured at 340 nm for 3 min and results expressed as nmol min^{-1} mg^{-1} of protein, using a molar extinction coefficient of 6.22×10^3 $M.cm^{-1}$. Aerobic and anaerobic pathways were also assessed through LDH/IDH ratio.

Cellular energy allocation (CEA) is an integrative methodology to assess responses to exposure in stressful scenarios by quantifying organismal energetic tradeoffs (available energy and energy consumption) based on the measurements of lipid, carbohydrate and protein content, and ETS activity. A decline in CEA indicates a reduction in available energy and/or higher energy expenditure and was calculated as in Verslycke et al., (2004a, 2004b): CEA = *Ea*/*Ec*, where *Ea* (available energy) = carbohydrate + lipid + protein (mJ.mg ww^{-1}), and *Ec* (energy consumption) = ETS activity (mJ h^{-1} mg ww^{-1}). Following De Coen and Janssen, (2003, 1997), total content of carbohydrates, lipids and proteins were measured and transformed into energetic equivalents using enthalpy combustion (39.5 kJ g^{-1} lipid, 24 kJ g^{-1} protein, 17.5 kJ g^{-1} glycogen, respectively). From the 300 µL aliquoted for total protein and carbohydrate measurements, proteins were precipitated with the addition of 100 µL of 15% trichloroacetic acid (TCA) following incubation at -20ºC for 10 min. After a centrifugation at 1000 g for 10 min, the resulting supernatant was used for the total carbohydrate content measurement. The remaining pellet was resuspended in 500 µL NaOH (1 M), incubated at 60ºC for 30 min and then neutralized with HCl (1.67 M). Total protein content was then quantified by the Bradford (1976) method measuring absorbance at 600nm and using bovine serum albumin as standard, as described above. Total carbohydrate content was determined by adding 50 µL of 5% phenol and 200 µL of concentrated H_2SO_4 to 50 µL of the supernatant fraction obtained before. After 30

min incubation at 20ºC, absorbance was measured at 492 nm and carbohydrates quantified using glucose as standard De Coen and Janssen, (1997). Lipids were extracted from a different homogenate aliquot (300 µL), by adding 250 µL chloroform, 250 µL methanol and 125 µL Milli-Q water (in a 2:2:1 proportion), adapted from Bligh and Dyer (1959). After centrifugation, 100 µL of organic phase were separated for lipid quantification following a reaction with 500 µL of H_2SO_4 at 200ºC for 20 min. After cooling, 1.5 mL of ultrapure water were added. The resulting absorbance was measured at 375 nm, and total lipid content quantified using tripalmitin as standard. Results were expressed as mJ mg wet weight^{-1}.

ETS activity in the mitochondria is a measurement of the cellular energy consumption (oxygen consumption rate) and can be determined following the method of De Coen and Janssen (1997), by adding 150 µL of a solution containing 0.13 M Tris, 0.3 % (v/v) Triton X-100, 1.68 mM NADH and 0.25 mM NADPH, followed by the addition of 100 µL of a 8.03 mM INT (p-iodo-nitro-tetrazolium) solution to the samples (50 µL). Change in absorbance was followed at 490 nm over a 3 min period. The oxygen consumption was then calculated using a stochiometrical relationship: 2 µmol of formazan formed = 1 µmol of oxygen consumed. The oxygen consumption rate was then converted into the energetic equivalent of 480 kJ.mol O_2^{-1} for average carbohydrate, lipid, and protein consumption combinations (Gnaiger, 1983).

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Figures and statistical analysis results

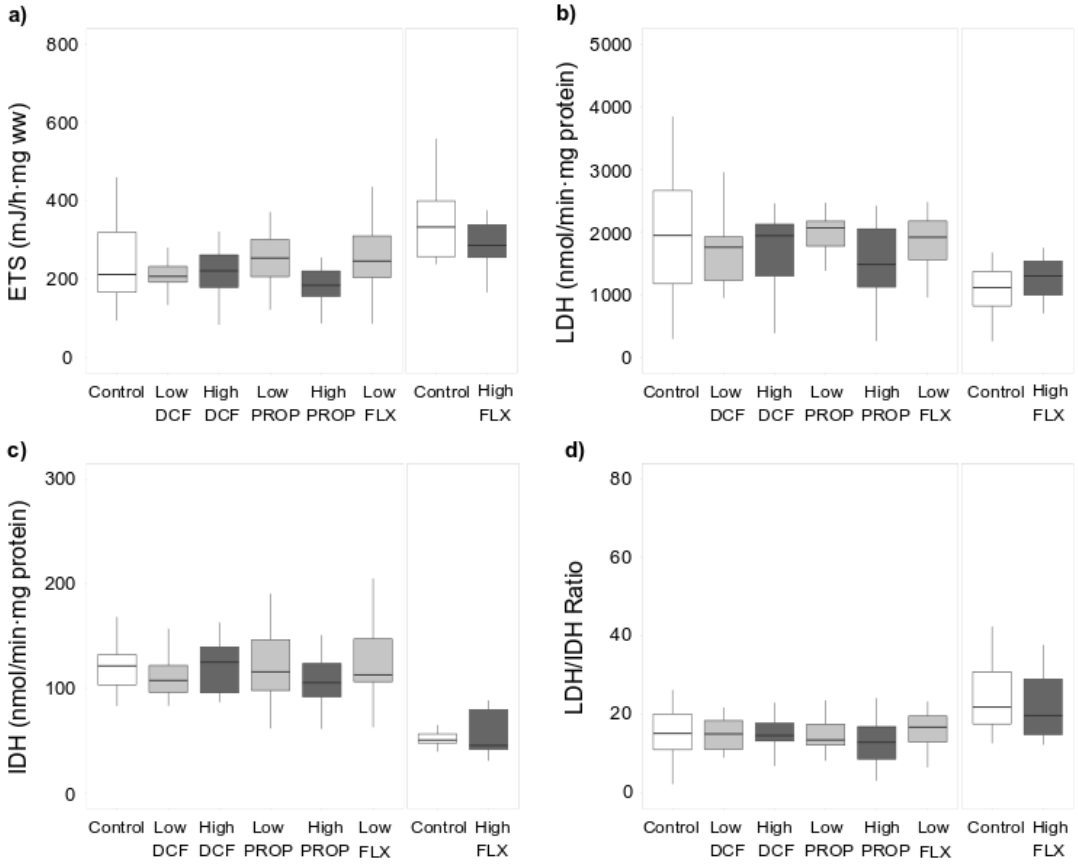

Figure A 3.1. Biomarker responses of *A. regius* juveniles after exposure to low (light grey) and high (dark grey) concentrations of diclofenac (DCF), propranolol (PROP) and fluoxetine (FLX). Boxplots with median, 25th and 75th percentile (upper and lower whiskers represent 1.5 times the interquartile range (IQR) of maximum and minimum values, respectively) of responses measured in the heart: a) electron transport system (ETS) activity, b) lactate dehydrogenase (LDH) activity, c) isocitrate dehydrogenase (IDH) activity and d) LDH/IDH ratio. N=22-24 juveniles per treatment, except for FLX high (N=15) and respective control (N=10).

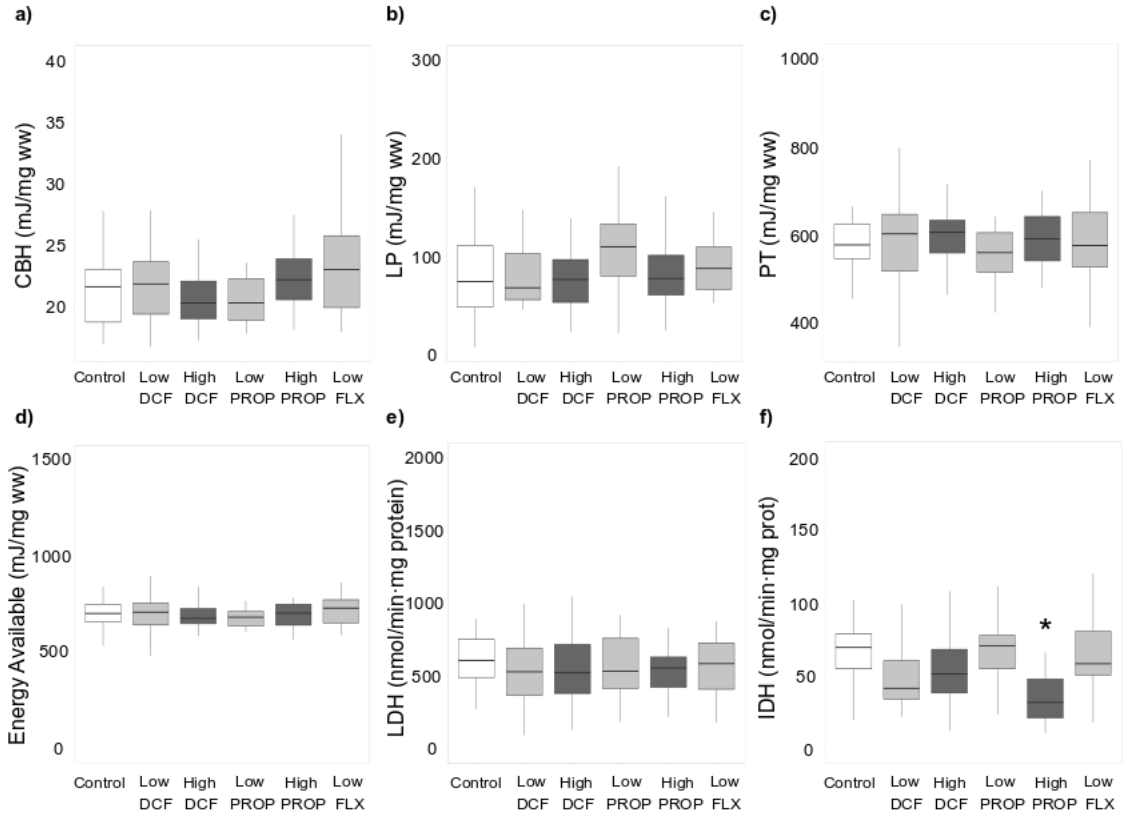

Figure A 3.2. Biomarker responses of *A. regius* juveniles after exposure to low (light grey) and high (dark grey) concentrations of diclofenac (DCF), propranolol (PROP) and fluoxetine (FLX). Boxplots with median, 25th and 75th percentile (upper and lower whiskers represent 1.5 times the interquartile range (IQR) of maximum and minimum values, respectively) of responses measured in the muscle: a) total carbohydrates (CBH), b) total lipid content (LP), c) total protein content (PT), d) energy available (EA), e) lactate dehydrogenase (LDH) activity and f) isocitrate dehydrogenase (IDH) activity. Asterisks indicate significant differences between treatments and respective controls. N=22-24 juveniles per treatment, except for FLX high (N=15) and respective control (N=10).

Table A 3.2. Summary of PERMANOVA and Mann-Whitney-Wilcoxon test results, testing for differences in *Argyrosomus regius* responses between treatments, after exposure to diclofenac (DCF), propranolol (PROP) and fluoxetine (FLX).

			Diclofenac (DCF)					Propranolol (PROP)					Fluoxetine (FLX)		
Response	**Tissue**	**Source**	**df**	**SS**	**MS**	**Pseudo-F**	**P(perm)**	**df**	**SS**	**MS**	**Pseudo-F**	**P(perm)**	**Treatment**	**W**	***p*-value**
Lt		Treatment	2	9.6E-02	4.8E-02	0.09	0.9170	2	2.3E-01	1.1E-01	0.21	0.8150	Low	333	0.1309
		Res.	66	3.4E+01	5.2E-01			55	2.9E+01	5.3E-01			High	119.5	0.0144
Wt		Treatment	2	1.8E+00	9.1E-01	0.44	0.6650	2	1.7E+00	8.4E-01	0.35	0.6970	Low	322	0.2058
		Res.	66	1.4E+02	2.0E+00			66	1.6E+02	2.4E+00			High	132	0.0017
K		Treatment	2	1.6E-02	7.9E-03	1.49	0.2210	2	2.2E-02	1.1E-02	1.32	0.2580	Low	230.5	0.4680
		Res.	66	3.5E-01	5.3E-03			66	5.6E-01	8.4E-03			High	103	0.1289
PT	Muscle	Treatment	2	3.4E+03	1.7E+03	0.25	0.7910	2	9.7E+03	4.9E+03	1.04	0.3650	Low	240	0.9722
		Res.	64	4.4E+05	6.9E+03			63	3.0E+05	4.7E+03					
LP	Muscle	Treatment	2	4.4E+02	2.2E+02	0.19	0.8200	2	8.3E+03	4.1E+03	2.39	0.1020	Low	125	0.3687
		Res.	57	6.5E+04	1.1E+03			60	1.0E+05	1.7E+03					
CBH	Muscle	Treatment	2	1.6E+01	8.0E+00	0.96	0.3920	2	3.3E+01	1.7E+01	2.62	0.0800	Low	183	0.1199
		Res.	65	5.4E+02	8.3E+00			61	3.9E+02	6.4E+00					
EA	Muscle	Treatment	2	1.6E+03	8.2E+02	0.12	0.8840	2	5.1E+03	2.6E+03	0.48	0.6230	Low	137	0.8249
		Res.	56	3.9E+05	7.0E+03			55	2.9E+05	5.3E+03					
IDH	Muscle	Treatment	2	3.4E+03	1.7E+03	3.21	0.0520	2	1.4E+04	7.0E+03	14.68	0.0010	Low	294	0.5201
		Res.	66	3.5E+04	5.2E+02			66	3.2E+04	4.8E+02					

	Heart	Treatment	2	1.5E+03	7.5E+02	1.48	0.2300	2	1.7E+03	8.6E+02	0.95	0.4070	Low	205	0.8613
		Res.	60	3.0E+04	5.1E+02			55	5.0E+04	9.0E+02			High	41	0.5516
LDH	Muscle	Treatment	2	7.0E+04	3.5E+04	0.72	0.5030	2	2.9E+04	1.5E+04	0.39	0.6550	Low	296	0.4922
		Res.	64	3.1E+06	4.8E+04			65	2.4E+06	3.8E+04					
	Heart	Treatment	2	6.4E-03	3.2E-03	0.07	0.9410	2	2.9E+06	1.4E+06	2.26	0.1070	Low	206	0.9687
		Res.	57	2.7E+00	4.8E-02			48	3.0E+07	6.3E+05			High	59	0.9748
ETS	Muscle	Treatment	2	8.1E+02	4.0E+02	3.88	0.0250	2	6.4E+02	3.2E+02	3.06	0.0610	Low	211	0.4779
		Res.	62	6.5E+03	1.0E+02			64	6.7E+03	1.1E+02					
	Heart	Treatment	2	1.9E-02	9.3E-03	0.38	0.6730	2	3.5E+04	1.7E+04	2.52	0.0880	Low	177	0.4143
		Res.	59	1.4E+00	2.4E-02			55	3.8E+05	6.9E+03			High	59	0.2775
LDH/IDH ratio	Muscle	Treatment	2	8.4E+01	4.2E+01	3.17	0.0530	2	1.2E+00	6.0E-01	19.41	0.0010	Low	211	0.9691
		Res.	55	7.3E+02	1.3E+01			60	1.8E+00	3.1E-02					
	Heart	Treatment	2	5.3E-01	2.6E-01	0.01	0.9870	2	3.9E+01	2.0E+01	0.52	0.5850	Low	157	0.8188
		Res.	53	1.4E+03	2.7E+01			42	1.6E+03	3.8E+01					
CEA	Muscle	Treatment	2	1.4E+02	7.1E+01	5.11	0.0050	2	8.3E+01	4.2E+01	2.08	0.1240	Low	152	0.3699
		Res.	50	7.0E+02	1.4E+01			52	1.0E+03	2.0E+01					
SOD	Liver	Treatment	2	4.9E-02	2.5E-02	2.80	0.0720	2	1.9E+01	9.7E+00	0.28	0.7690	Low	257	0.5393
		Res.	66	5.8E-01	8.8E-03			61	2.1E+03	3.4E+01			High	13	0.0499
CAT	Liver	Treatment	2	8.0E+02	4.0E+02	3.00	0.0580	2	1.1E+00	5.4E-01	0.004	0.9980	Low	206.5	0.2963
		Res.	66	8.8E+03	1.3E+02			66	8.7E+03	1.3E+02			High	14	0.0650

GST	Liver	Treatment	2	5.2E-03	2.6E-03	0.17	0.8450	2	2.3E+03	1.2E+03	2.78	0.0890	Low	242	0.8134
		Res.	66	1.0E+00	1.6E-02			66	2.8E+04	4.2E+02			High	59	0.0274
EROD	Liver	Treatment	2	4.6E-04	2.3E-04	0.00	0.9990	2	5.5E+02	2.7E+02	1.29	0.2860	Low	211	0.8228
		Res.	61	6.4E+00	1.1E-01			65	1.4E+04	2.1E+02			High	60	0.0207
LPO	Muscle	Treatment	2	4.2E-02	2.1E-02	2.29	0.1100	2	1.3E-03	6.6E-04	1.38	0.2650	Low	251.5	0.6090
		Res.	62	5.8E-01	9.3E-03			61	2.9E-02	4.8E-04					
	Liver	Treatment	2	3.2E-04	1.6E-04	0.29	0.7710	2	2.0E-03	1.0E-03	1.82	0.1670	Low	357	0.0063
		Res.	64	3.6E-02	5.6E-04			63	3.5E-02	5.5E-04			High	6	0.0047
DNAd	Muscle	Treatment	2	1.2E-02	6.2E-03	0.20	0.8020	2	1.6E-01	7.8E-02	3.41	0.0460	Low	273.5	0.1866
		Res.	65	2.0E+00	3.1E-02			64	1.5E+00	2.3E-02					
	Liver	Treatment	2	8.7E-02	4.3E-02	0.48	0.6380	2	4.4E-01	2.2E-01	2.62	0.0820	Low	276	0.5963
		Res.	64	5.8E+00	9.1E-02			63	5.3E+00	8.4E-02			High	12	0.0418
AChE	Brain	Treatment	2	1.5E+03	7.7E+02	0.60	0.5490	2	1.4E+03	7.0E+02	0.72	0.4930	Low	285	0.4782
		Res.	65	8.3E+04	1.3E+03			63	6.1E+04	9.6E+02			High	51	0.5910

Table A 3.3. Summary of Welch's t-test results, testing for differences in *Argyrosomus regius* growth (G) and bioconcentration between treatments, after exposure to diclofenac (DCF), propranolol (PROP) and fluoxetine (FLX).

		Diclofenac (DCF)			**Propranolol (PROP)**			**Fluoxetine (FLX)**		
	Treatment	**t**	**df**	***p*-value**	**t**	**df**	***p*-value**	**t**	**df**	***p*-value**
G	Low	1.954	3.6	0.130	-0.4	4.0	0.731	0.6	4.0	0.596
	High	-0.071	3.9	0.947	1.2	3.8	0.300	6.2	3.4	0.006
Bioconcentration	Low	N/A			-15.3	5.0	2.15E-05	-77.2	7.0	1.61E-11
	High	N/A			-23.5	6.0	3.86E-07	-18.1	2.0	0.003

Table A 3.4. Spearman correlation results (correlation coefficients r and p-values as asterisks; * p < 0.05, ** p < 0.01, *** p < 0.001), testing for correlations among *Argyrosomus regius* responses after exposure to fluoxetine (FLX).

	Lt (cm)	Wt (g)	K	LDH muscle	IDH muscle	LDH/IDH ratio muscle	ETS muscle	CBH muscle	LP muscle	PT muscle	EA muscle	CEA muscle	LPO muscle	DNAd muscle	LDH heart	IDH heart	LDH/IDH ratio heart	ETS heart	SOD liver	CAT liver	GST liver	EROD liver	LPO liver	DNAd liver	AChE brain
Lt (cm)	1																								
Wt (g)	0.93 ***	1																							
K	-0,11	0,23	1																						
LDH muscle	-0,06	0,01	0,16	1																					
IDH muscle	0,08	0,11	0,03	0,2	1																				
LDH/IDH ratio muscle	-0,13	-0,08	0,11	0.66 ***	-0.38 *	1																			
ETS muscle	-0.4 **	-0.34 *	0,18	-0,23	-0,05	-0,22	1																		
CBH muscle	0,03	0,07	-0,08	0,12	0,29	-0,03	-0,15	1																	
LP muscle	-0,07	-0,14	0,01	-0,07	0	0,11	0,24	0,02	1																
PT muscle	-0,06	-0,14	-0,15	-0,02	-0,07	-0,05	-0,02	-0,15	-0,11	1															
EA muscle	0,2	0,1	-0,14	-0,12	-0,07	-0,03	-0,11	0,02	0.45 **	0.75 ***	1														
CEA muscle	0,22	0,12	-0,3	0,18	0	0,25	-0.89 ***	0,23	-0,15	0.36 *	0.44 *	1													
LPO muscle	-0.45 **	-0.42 **	0,15	0,04	-0,23	0,22	0,07	-0,27	0,32	0,2	0	-0,12	1												
DNAd muscle	0,16	0,07	-0,04	0,26	0,1	-0,06	-0,09	-0,13	-0,26	0,02	-0,19	0,04	-0,03	1											
LDH heart	-0,06	-0,09	-0,01	0,22	-0,21	0,2	-0,02	0,14	-0,04	0,14	0,07	0,28	0,12	0,15	1										
IDH heart	0,2	0,24	0,19	-0,1	0,25	-0,13	0,05	0,05	-0,04	0,15	0,17	0,13	0,11	-0,05	0.45 ***	1									
LDH/IDH ratio heart	-0,11	-0,12	0,01	0,24	-0.42 *	0,32	0,08	-0,04	0,03	-0,09	-0,09	0,03	0,03	0,12	0.37 **	-0.56 ***	1								
ETS heart	-0.32 *	-0.32 *	-0,01	0,11	0,15	0,32	-0,15	0,03	0,14	0,01	0,1	0,2	0,26	-0,15	-0,06	-0,13	0,03	1							
SOD liver	0,03	-0,04	-0,13	0,07	0	-0,07	-0.34 *	0,02	-0,06	-0,23	-0,25	0,28	0,04	0,17	0,27	0,11	-0,01	-0,21	1						
CAT liver	0,04	0,03	-0,08	0,06	0,17	-0,09	-0,26	0,12	0,19	0,03	0,14	0,13	0,04	0,05	0,03	-0,15	-0,03	-0,23	0,22	1					
GST liver	0,12	0,16	0,2	0,02	0,27	-0,24	-0,04	-0,15	0	0,21	0,15	0,12	-0,24	0,12	0.3 *	0.29 *	-0,08	-0,15	-0,02	0,11	1				
EROD liver	0.57 ***	0.61 ***	0,07	-0,01	-0,01	0,01	-0,16	0,18	-0,08	0,02	0,09	0,13	-0,31	-0,06	0,08	0.3 *	-0,18	-0,23	-0,18	-0,25	0,2	1			
LPO liver	-0,03	-0,06	-0,05	0.42 **	0,21	0,25	-0,27	-0,01	-0,12	-0,16	-0,25	0,21	0,02	0.45 **	-0,17	-0.32 *	0,21	0	0.3 *	0,03	-0.36 **	-0,14	1		
DNAd liver	-0.41 ***	-0.46 ***	-0,14	-0,12	0,01	0	0.55 ***	-0,29	0,12	-0,15	-0,29	-0.63 ***	0,23	-0,03	-0.31 *	-0.27 *	0,09	0.34 *	-0,22	-0,09	-0.4 **	-0.56 ***	0,24	1	
AChE brain	-0,14	-0,11	-0,01	-0,1	0.45 **	-0,28	0,11	0,17	0,02	0,04	-0,03	-0,08	0,14	-0,24	-0,05	0.08	-0,17	0,13	-0,03	0,04	-0,07	-0,25	-0,05	0,01	1

Table A 3.5. Spearman correlation results (correlation coefficients r and p-values as asterisks; * p < 0.05, ** p < 0.01, *** p < 0.001), testing for correlations among *Argyrosomus regius* responses after exposure to diclofenac (DCF).

	Lt (cm)	Wt (g)	K	LDH muscle	IDH muscle	LDH/IDH ratio muscle	ETS muscle	CBH muscle	LP muscle	PT muscle	EA muscle	CEA muscle	LPO muscle	DNAd muscle	LDH heart	IDH heart	LDH/IDH ratio heart	ETS heart	SOD liver	CAT liver	GST liver	EROD liver	LPO liver	DNAd liver	AChE brain
Lt (cm)	1																								
Wt (g)	0.94 ***	1																							
K	-0.33 **	-0,06	1																						
LDH muscle	-0,06	-0,06	-0,07	1																					
IDH muscle	0,04	0,01	-0,09	0.36 **	1																				
LDH/IDH ratio muscle	0,04	0,07	-0,06	0.49 ***	-0.35 **	1																			
ETS muscle	-0,13	-0,06	0,19	-0,08	-0,15	0,11	1																		
CBH muscle	0,07	0,02	-0,18	0,02	-0,05	-0,01	-0,04	1																	
LP muscle	0,16	0,18	-0,02	0,06	0,19	-0,05	0,01	-0,04	1																
PT muscle	-0,07	-0,13	-0,14	-0,1	-0,19	0,21	-0.03	0.36 **	-0,08	1															
EA muscle	-0,03	-0,08	-0,16	0,02	-0,07	0,21	-0,02	0.41 **	0.28 *	0.87 ***	1														
CEA muscle	-0,07	-0,18	-0,26	0,03	0,1	-0,08	-0.87 ***	0,19	-0,14	0.29 *	0.32 *	1													
LPO muscle	-0,06	-0,08	-0,03	-0,09	-0,15	0,08	0,1	0,05	0,06	0,19	0,15	-0,02	1												
DNAd muscle	-0,16	-0,17	-0,01	0.34 **	-0,04	0,24	-0,18	0,02	-0,24	0,06	0,01	0,15	-0,02	1											
LDH heart	-0,17	-0,2	-0,01	0,04	-0,01	-0,1	-0,2	0,09	-0,07	-0,05	-0,12	0.34 *	0,23	-0,08	1										
IDH heart	-0,06	-0,11	-0,15	-0,12	0,22	-0,09	-0,05	-0,23	-0,05	-0,17	-0,21	0,11	-0,09	-0,2	0.31 *	1									
LDH/IDH ratio heart	-0,06	-0,06	0,15	0	-0,11	-0,12	-0,1	0,12	-0,03	0,06	0,04	0,25	0.28 *	0,04	0.82 ***	-0,21	1								
ETS heart	-0,06	-0,13	-0,05	-0,1	0,03	-0,04	-0,26	-0,03	0,15	-0,03	0,08	0,25	-0,13	-0,03	0.48 ***	0.34 **	0,2	1							
SOD liver	0,01	0,03	0	-0,1	0,2	-0.28 *	0,01	0,09	0,05	0,04	0,02	0,08	-0,05	-0,11	0,15	0	0,23	-0,03	1						
CAT liver	0,19	0.25 *	-0,01	0,11	0.31 **	-0,14	-0,06	-0,14	0,14	-0,19	-0,16	-0,06	-0,14	-0,2	0,03	0,17	-0,13	-0,11	0.31 **	1					
GST liver	0.29 *	0.31 **	-0,07	-0,06	0,15	-0,09	0,12	-0,04	0,08	0,07	0,06	-0,2	0,07	-0.34 **	-0,06	-0,04	-0,06	0	0,12	0.3 *	1				
EROD liver	0.32 **	0.39 **	0,01	-0,03	-0,12	0,13	-0,08	0,2	-0,21	0,15	0,04	-0,07	0,11	0,02	-0,15	-0,25	-0,01	-0,18	0	0,02	0.27 *	1			
LPO liver	-0,08	-0,08	0	0,09	-0,06	0,12	-0.32 *	-0,18	-0,1	-0,03	-0,08	0.27 *	0,22	0.38 **	0,07	0,06	0,14	-0,01	0,02	-0,13	-0.47 ***	0	1		
DNAd liver	-0.32 **	-0.3 *	0,01	0,16	0,12	0,14	0,16	-0,22	0,01	-0,19	-0,13	-0,01	0,17	0,04	-0,01	0,2	-0,1	0,05	-0,24	-0,14	-0.28 *	-0.38 **	0,11	1	
AChE brain	-0.1	-0,12	-0,06	0,11	0,23	-0,01	0,08	-0,07	0,07	0,11	0,07	0,03	0,2	-0,01	0,17	0,08	0,23	-0,14	-0,07	-0,13	-0,04	-0.27 *	0	0,15	1

Table A 3.6. Spearman correlation results (correlation coefficients r and p-values as asterisks; * p < 0.05, ** p < 0.01, *** p < 0.001), testing for correlations among *Argyrosomus regius* responses after exposure to propranolol (PROP).

	Lt (cm)	Wt (g)	K	LDH muscle	IDH muscle	LDH/IDH ratio muscle	ETS muscle	CBH muscle	LP muscle	PT muscle	EA muscle	CEA muscle	LPO muscle	DNAd muscle	LDH heart	IDH heart	LDH/IDH ratio heart	ETS heart	SOD liver	CAT liver	GST liver	EROD liver	LPO liver	DNAd liver	AChE brain
Lt (cm)	1																								
Wt (g)	0.92 ***	1																							
K	-0.37 **	-0,03	1																						
LDH muscle	-0,02	-0,1	-0,09	1																					
IDH muscle	-0,06	-0,14	-0,2	0.33 **	1																				
LDH/IDH ratio muscle	0,14	0,19	0,14	0.41 ***	-0.6 ***	1																			
ETS muscle	-0,21	-0,1	0,21	-0,2	-0.27 *	0,11	1																		
CBH muscle	0,07	-0,01	-0,21	0,04	0,02	0,19	-0,15	1																	
LP muscle	0	-0,01	-0,04	-0,03	0,06	-0,14	0,11	0,02	1																
PT muscle	-0,13	-0,11	0,07	0	0,08	0,01	-0,18	0,06	-0,15	1															
EA muscle	-0,12	-0,06	0,16	-0,03	0,1	-0,03	-0,15	0,19	0.35 **	0.78 ***	1														
CEA muscle	-0,01	-0,09	-0,1	0,11	0.3 *	-0,17	-0.91 ***	0.3 *	-0,09	0.46 ***	0.48 ***	1													
LPO muscle	-0,09	-0,12	0,07	-0,19	-0,2	0,05	0,06	0,06	0,24	0,11	0,07	-0,04	1												
DNAd muscle	0,08	0,02	-0,12	0,27	-0,13	0,23	0,04	-0,02	-0,12	0,01	-0,15	-0,15	-0,13	1											
LDH heart	-0.3 *	-0.37 **	-0,1	0.24 *	0,16	-0,11	-0,09	-0,01	-0,05	0,1	0,06	0,2	-0,08	-0,02	1										
IDH heart	-0,15	-0.16	0,09	-0,11	0,07	-0,04	0,23	0,04	0,07	-0,06	0,03	-0,08	0,05	-0,11	0,02	1									
LDH/IDH ratio heart	-0,29	-0.34 *	0,03	0,27	0,08	-0,06	-0,15	-0,17	-0,09	0,15	0,13	0,27	0,07	0,19	0.87 ***	-0,21	1								
ETS heart	-0,09	-0,15	-0,09	-0,02	0,07	-0,02	-0,04	-0,11	0.3 *	-0,13	0	0,1	0,02	-0,12	0.37 **	0,19	0.35 *	1							
SOD liver	-0,08	-0,13	-0,17	-0,06	0	-0,14	-0,16	0	0,15	0,02	0,13	0.32 *	0,14	-0,07	-0,08	0,04	0,08	0,24	1						
CAT liver	0,01	0,01	-0,06	-0,03	-0,02	-0,03	0,07	0,1	0,11	-0,04	0,08	-0,13	0,02	0,11	-0,01	-0,03	0,03	-0,08	0,12	1					
GST liver	0,1	0,11	0,03	-0,1	0,03	-0,04	0,06	-0,07	0,06	-0,14	-0,13	-0,13	-0,07	0,02	0,13	0,11	-0,01	0,1	0	0,23	1				
EROD liver	0,18	0,23	0,08	-0,15	0,03	-0,03	-0,12	0,1	0,05	-0,15	-0,12	0,09	0,02	-0,03	-0,1	0	-0,1	0,03	-0,17	-0,05	0.25 *	1			
LPO liver	-0,14	-0,1	0,07	0,14	-0,05	0,12	-0,14	-0.28 *	-0.39 **	0,07	-0,14	0,01	-0,1	0,18	-0,03	-0,04	0,16	-0,05	0,04	-0,14	-0.47 ***	-0,05	1		
DNAd liver	-0.46 ***	-0.51 ***	-0,04	0,14	0.27 *	-0,16	0,01	-0,09	-0,09	0,1	0,06	0,04	-0,19	-0,13	0,19	0,12	0,11	0,04	-0,19	-0,21	-0,22	-0.3 *	0,13	1	
AChE brain	-0,05	-0,1	-0,09	-0,08	0,12	-0,17	-0,12	0,07	-0,02	-0,04	-0,09	0,02	0,15	-0,25	0	-0,07	0,06	-0,14	-0,01	0,23	-0,03	-0,16	0,04	-0,03	1