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Mestre em Biologia Marinha

Climate change and emerging chemical contaminants in marine organisms: Bioaccumulation, ecotoxicology and public health impacts

Dissertação para obtenção do Grau de Doutor em
Ambiente e Sustentabilidade

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ACKNOWLEDGMENTS

"All your dreams can come true if you have the courage to pursue them. Remember that this whole thing started with a dream and a mouse…"

Walter Disney

Some dreams start with a mouse… others start with a fish!

If ten years ago someone had told me that today I would be on the verge of holding a PhD degree, I just would not have believed it! At first, simply because it was not part of my plans and, later, because I just didn't feel I was good enough to do it. Those that are closest to me know how hard it was to start this journey, how many times I felt like quitting the dream, and the rocky path I had to go through before getting where I wanted. Yet, things always seem a lot easier when we are surrounded by great and loving people that never stop believing, guiding, supporting and pushing us to go further. Some may say that we come across people in our lives merely by chance, fate or luck. If that is so, at this point, I feel like one of the luckiest people on hearth because, for four years, I was surrounded by the best, and to them I owe this dream come true!

Since the beginning of this journey, I always knew exactly who I wanted to thank when the time came. But, finding the right words to do it… that was the hard part! Four years have passed in a blink of an eye and, still, I feel that there are not enough words to describe all my gratitude and feelings for these people. Doing it in English further increased the challenge!

In hopes that this will get easier as I go along, I will start by thanking the **EU project ECsafeSEAFOOD** (grant agreement 311820) for supporting my PhD research work straight from the beginning (and even before engaging in this PhD journey), and this is where it all started, back in 2013 … We all know that it is part of the protocol to acknowledge all funding institutions. However, I really want to thank this project, not just for buying the reagents/equipment that were necessary for my research (nor for paying for my salary during almost 2 years), but for all the opportunities that it gave me during these four years. Being part of ECsafeSEAFOOD allowed me to meet many great scientists, become acquainted and interested in new research topics and methodologies, visit national and international institutions and establish several partnerships with them (and I believe that these partnerships can go far beyond these four years of PhD)… Altogether, being part of this big and ambitious project definitively enriched my PhD journey and made me grow a lot as a scientist and as a person.

Then, also as a "must do", I want to acknowledge **The Portuguese Foundation for Science and Technology (FCT)**, not only for granting me with a PhD fellowship (SFRH/BD/103569/2014) which payed for my salary and tuition (from August 2015 until now), but also for changing the way PhD candidates are evaluated and, therefore, recognizing my merit and strong will to pursue this dream, after rejecting my PhD application for two consecutive years. By looking at PhD candidates from all different angles, people are no longer mainly defined by a

number (i.e. a college grade), but rather by what they have made out of it (i.e. their experience and CV). I may be wrong, but, personally, I think it is a fairer and broader way of judging people and deciding on their future…

This "leaves the door opened" to talk about my two amazing supervisors, Doctors **Mário Sousa Diniz** and **Rui Rosa**, who never stopped believing I could do this, regardless of the consecutive application rejections, and received me in their research groups with "opened arms", almost like fathers do. And because they are amazing mentors in such distinct ways, I have to talk about them separately.

Mário, you became my "lucky charm", as it was only when I met you and you agreed to be my supervisor that I finally got my PhD grant. So, just because of that I will be forever thankful to you. I will never forget your words when I phoned you to tell the good news: *"The merit is all yours…"*. Yet, there's much more to say about you: First, I have to say that you introduced me to my true research passion, i.e. ecotoxicology (everything that I know about it, I owe to you), and that was a life-changing moment that I will take with me forever. Second, if I had to define you in two words those would be kindness and reassurance, and I was so blessed to have you as my mentor. You always had a nice word to say, a positive reinforcement and, during hard times, you had the gift of calming me down, simplifying/demystifying what scared me and making me realize that, at the end, everything would be ok. Finally, your "non-sense stories", along with your serious and so particular sense of humour made me laugh so many times, and everyone knows how much we need good laughs during our PhD journey…

Rui, to me, you were that kind of father that tells a child to "*climb on a chair and go for it"*, instead of just picking up the glass from a high shelf and handing it on its hands. But, if the child falls, you are there to straighten up the chair while saying *"try again, but this time do it right"*. When the child finally manages to get the glass without falling from the chair, there you are, clapping your hands and saying out loud: *"Congratulations! I knew you could do it, I'm so proud of you…"*. I believe that this is the kind of "parenting" that makes us grow in life and, so, I have to thank you for all that you made me grow as a scientist. For all the "inflamed arguments", criticism, motivation to push myself harder and think "outside the box" and, especially, for letting me know how proud of me you were, I thank you! You may not have simplified this PhD journey, but you definitely made it more challenging and great.

Mário and **Rui**, as much as I may be happy to conclude my PhD journey, I cannot help feeling sad for having to leave you two, almost like a baby bird that has to leave its parents nest and fly on its own! That "solo-flight" is still kind of scary to me… Thank you for inspiring me in so many different ways, for embracing me in your teams and for being at my side during all these years. I hope life grants me the opportunity to keep working with you, for many, many years to come…

Yes, it's true that my two supervisors welcomed me in their research teams… but, it was only because of all the great people that are part of these teams that I felt cherished and happy to go to work every day. In this way, I have to thank the "Wood-Twins", **Diana** and **Carolina** (my IBRs-Gurus), from UCIBIO, REQUIMTE, as well as (my apologies if I forget someone, but you guys are too many "manas"…) **Repolhito** (my Profilux-Guru), **Migas**, **Kuka**, **Ritinha**, **Seratoninas**, **Dúdú**, **Mana-Beijinhos**, **Pims**, **Vasquito**, **Grilo**, **Cyrne** (my old-time college friend), **fainting-Cat** and **Mummy-M.Rita** from MARE, for always being willing to help me with a smile on their faces (except Repolho), every time I've cried for help (and every time I had to go to that horrible "seawater-pumping-house")! I see in all of you a friend!

As for the IPMA team, I have to devote a bit more time to talk about these colleagues-friends, as most of my working time (and not only) was spent with them. Meeting them was my ultimate proof that, indeed, *"it's the people that make the places, and not the places that make the people"*! When I first started working at IPMA (and this was back in 2006), I didn't like it at all! The working environment at the lab was so heavy and competitive… People just wouldn't smile, help, talk with each other or share experiences and, therefore, becoming friends was, obviously, unthinkable. Thus, for a long time, I used to feel that it was torture to wake up to go to work every day. But then, slowly, month by month, year by year, as some went away and others came along, things started to become lighter, better, and the lab was, for the first time in my life, a happy place! The people that enabled that drastic (and for the better) change are my today's best-friends **Patitas** (my first friend at IPMA), **Vericas** (my old-time "partner in crime", room and college mate), **Xoxon** (my old-time college and scuba-diving mate), **Carol** (my right-arm in Guia) and **Martinha** (my most recent colleague and friend). Thank you for making me feel happy to go to work, giving me the strength I needed to pursue this dream and helping me in each and every tortuous trial and sampling (followed by all those well-deserved lunch-times at MacDonald's). Thank you for all the great moments (dinners, holiday-trips, parties), all the laughs-out-loud and, simply, for being my unconditional friends! A final note: Xoxon, I'm sorry for every time I tried to pull you away from your dream. I know I've said that I was doing so because I felt that you wouldn't be happy where you are now, and that was true. Though, only partially true (like, 20%)… The other 80% was just me being terrified with the idea of losing you again. I need you so much in my life and, now, I miss you so much at IPMA (and outside of it). Please, try to accomplish that dream as fast as you can and, once you've accomplished it, just come back to us, ok? We still have a great project to submit together, remember?

Still within IPMA's team, I would like to mention one last person… I wasn't sure where to place her in this Acknowledgments Section, because she started off as my teacher, quickly she became my mentor and leader, then she became a friend and, later, she became like a "mother-figure" to me (and to so many of us at IPMA). **Eng[a] Leonor**, thank you for all the experience, knowledge and wisdom you are always willing to share with us. Thank you for all the kindness,

selflessness and support, like only mothers have. Someone once said: *"try to find a job that you love and you won't have to work a single day in your life"* and, so, I thank you for making us feel passionate about our work, for inspiring us every day and making us dream of becoming the great scientist and team-leader that you are.

Like I said above, today, I feel I've been blessed because life has granted me so many wonderful friends… In fact, they are so many that I could write a whole PhD thesis just on Acknowledgments… In brief, my friends "outside of work" include people that I know for less than 10 years, as well as those that have been part of my life for 30 years (and I'm only 35!). Starting with the most recent acquisitions, I want to thank **Shorty-knows-it-all**, **Rafa-mother-of-two** and, especially, **Raquel-Xoxonette** who has made me laugh so many times and backed me up in my worst days. Then, I have to thank my two dear friends I've kept since college, **Licas** and **Angelo-The-President**, for being part of my growing process, for sharing so many great moments and stories that will forever be in our memories (and some of those stories should stay there!) and for being like the two older brothers I didn't have… Moving on to high school times, I to want to thank my three "partners-in-crime" **CláuSol**, **Pimenta** and **Cristininha**, as well as our "body-guard" **Nuno Neto-Carequinha-Sr. Avariador**: we grew together, went on separate ways, became great in such distinct ways and, even though we are so different from each other, somehow, we managed to keep this amazing friendship alive throughout time… I thank you for that! And, at last (but not at least), I want to thank my forever-friend **Marta Patrício**: We started just as childhood neighbours and, step by step, she became like my older-sister, who I would look up to. Then, when I was 17 years old, I went to college in Faro while she stayed in Lisbon University and, so, we've been apart for some years (too many). For all this time, we had completely different experiences, routines, hobbies, goals, dreams… she is, perhaps, the friend that went on the most different direction and became the person that is the most different to me. But, when I came back, it was almost like time had stopped during the time we've been apart, and the friendship stayed the same, though more mature, honest and strong… Moreover, she (and, obviously, her husband, Rui, which is also a great guy and old-time friend) has recently presented me with the cutest girl and best God-daughter ever, my sweet baby-Matilde. For all of these things that made, for 30 years, my life so rich and colourful I thank you! May life (at least) triplicate the time of our friendship…

Finally, it's time to talk about family, about those who love me unconditionally, no matter how good or bad I do in life. These are the people that I owe my life to: they have taken care and protected me for many years, and, of course, they are the ones that know me the best… And how can you thank them for that? In the absence of words to appropriately transcribe the greatness of our love for each other, I usually try to express all my feelings for them through actions or simply by being there when they need me. Yet, because I have to put out some words about them in this

Acknowledgements Section, I will start by saying that, once again, I feel that life has blessed me! First, because I have the most tender, affectionate, patient, selfless, attentive and supportive mother one could ever have… that kind of **Mother** (like all should be) that abdicates things all the time to give them to her child instead! Second, because most people have one father, but not me! Life gave me two dads that are amazing in their own different ways: They have taught me different things and ways to behave in life, shared different experiences and contributed to my development as the balanced, fair and honest woman that I believe I became (or that, at least, I try to be…). Their only mistake was not to have given me any siblings. And, still, life detoured nature and gave me, anyway, a wonderful "sister from the heart", which is always there not only to annoy me and argue with me for the simplest and most non-sense things, but also to back me up and share the worst and the best times of our lives! **Mom**, **Dad**, **Matos** and **Vanessa**, thank you for being there and for loving me like you do! I love you to the moon and back!

Now, this may sound weird or even ridiculous but, somehow, I feel I also need to acknowledge my two dogs. During these last months, most of my time was spent at home, alone, completely dedicated to the writing of manuscripts and my PhD thesis. This phase was filled with moments of absolute silence, monotony and loneliness which made me feel depressed and, sometimes, like going crazy! Yet, such feelings were somewhat counteracted by the constant presence of my two dogs… simply because they bark all the time (and for no reason), they play and fight with each other, in the meantime, messing up the whole house, and they never hesitate to call for my attention and affection! **Twiggy** and **Scuba**, I thank you for insistently breaking the monotony of life, for forcing me to leave the house (and the computer, sometimes), for keeping me company while I was alone at home writing, for lighting up my days and, especially, for making me feel like the most special and unique person in the world! I'm sorry for not being so attentive, patient, playful and available during this phase, or even for yelling at you when you tried to distract me from my work… I know I wasn't the best dog-owner, like you deserve, but I promise that, from now on, I'll try my best to make up for all of those moments! As odd as this may seem, these are almost the same words I want to say to my husband. Throughout this whole PhD journey, my husband was my "main advisor/supervisor", my shelter, my number one believer and supporter! He taught me that "hard work pays off". He showed me how important it is to be persistent, to fight for what we want, to believe in ourselves and, especially, to always look at the bright side of life. Perhaps, he was the one that suffered the most while I was completely devoted to the PhD thesis and, so, I want to thank him for loving me and not quitting on me when I was less attentive, affectionate, sweet, as well as when I was in a really bad mood (and I was many, many times), complaining about my life. **António**, I promise you and our sweet boy **Afonso** the same thing I promised our dogs: from now on, all my efforts will be channelled towards making up to you for these years, during which I know I was not at my best. It's time to focus my attention in our needs as a family and, so, I'll try my best to make you as happy. I will not do so because it is my duty,

but because (even when it doesn't look like) I love you, and you two are the most important things in my life (as well as the dogs ☺)…

I dreamed this dream, but you made it possible. I dreamed it big, but you made it great. It might have been my dream in the first place but, at the end, it was your accomplishment. So, to you, **António**, I dedicate these 4 years of hard work brought together in the form of "dream come true". I hope I made you proud…

"I used to think that top environmental problems were biodiversity loss, ecosystem collapse and climate change. I thought that thirty years of good science could address these problems. I was wrong.

The top environmental problems are selfishness, greed and apathy, and to deal with these we need a cultural and spiritual transformation.

And we scientists don't know how to do that."

James Gustave Speth

RESUMO

A contaminação química e as alterações climáticas constituem dois dos maiores problemas ambientais relacionados com o aumento das actividades antropogénicas. Apesar de ambos desencadearem isoladamente impactos negativos nos ecossistemas marinhos e na segurança do pescado, pouco se sabe sobre as potenciais consequências da sua interação. Neste contexto, esta tese de doutoramento pretendeu avaliar os efeitos do aquecimento e/ou acidificação da água do mar na bioacumulação de diversos contaminantes químicos emergentes (ECCs; MeHg, iAs, DCF, VFX, TCS, Decs, TBBPA, PFOS e PFOA) e nas respostas ecotoxicológicas de dois grupos taxonómicos marinhos (peixes e bivalves). Em geral, o aquecimento facilitou a bioacumulação de compostos lipofílicos e persistentes (ex. MeHg, Decs e TBBPA), sugerindo que os riscos de exposição humana a estes compostos através do consumo de pescado serão maiores no futuro. Por outro lado, o aquecimento e/ou acidificação resultaram numa menor bioacumulação de compostos ionizáveis e menos persistentes (ex. iAs, VFX e TCS). Este decréscimo não significa necessariamente que os riscos para a saúde humana sejam menores, podendo implicar uma maior biotransformação de compostos na sua forma parental e, consequentemente, concentrações mais elevadas de metabolitos cujos efeitos toxicológicos (para animais e humanos) são ainda desconhecidos. Relativamente aos efeitos ecotoxicologicos, a exposição simultânea aos ECCs, aquecimento e acidificação traduziu-se, geralmente, em respostas mais severas (ao nível bioquímico, bem como na condição e comportamento animal) comparativamente às induzidas quando cada fator de stress atuou de forma isolada. Estes resultados sugerem que a exposição a ECCs num contexto de alterações climáticas irá, muito possivelmente, colocar à prova a resiliência dos organismos marinhos, nomeadamente daqueles que habitam zonas costeiras. Assim, as alterações climáticas irão representar um grande desafio à sustentabilidade e gestão das pescas e da aquacultura, sendo urgente desenvolver medidas de regulação, mitigação e adaptação à escala global.

Palavras-chave: contaminantes químicos emergentes; alterações climáticas; mecanismos de bioacumulação; ecotoxicologia; segurança do pescado.

ABSTRACT

Chemical contamination and climate change constitute two of the greatest environmental problems related with the increase of anthropogenic activities. Despite both factors acting alone can have negative effects at different levels of biological organization, as well as in seafood safety, the underlying interactions between them are still poorly understood. In this context, this PhD thesis aimed to assess the combined effects of seawater warming and/or acidification on the bioaccumulation of different emerging chemical contaminants (ECCs; MeHg, iAs, DCF, VFX, TCS, Decs, TBBPA, PFOS e PFOA) and ecotoxicological responses of two marine taxonomic groups (fish and bivalves). Overall, warming promoted the bioaccumulation of lipophilic and persistent ECCs (e.g. MeHg, Decs and TBBPA), suggesting increased risks of human exposure to these compounds through the consumption of contaminated seafood in tomorrow's ocean. Conversely, warming and/or acidification elicited lower bioaccumulation of ionisable and/or less persistent compounds (e.g. iAs, VFX and TCS). Yet, this trend may not necessarily represent lower human risks, as it may be associated with enhanced biotransformation of parental ECCs, potentially representing increased levels of metabolites for which the toxicological attributes (to both biota and humans) are still unknown. Regarding the ecotoxicological effects, overall, the simultaneous exposure to ECCs, warming and acidification promoted more severe responses (at the biochemical, animal condition and behavioural levels) than the ones elicited when each stressor acted in isolation. Such results reveal that the exposure to ECCs in a climate change context will likely defy the resilience of marine organisms, particularly those inhabiting coastal areas. Hence, climate change will greatly challenge the sustainability and management of fisheries and aquaculture resources, thus, calling for urgent regulatory, mitigation and/or adaptive actions at a global scale.

Keywords: emerging chemical contaminants; climate change; bioaccumulation mechanisms; ecotoxicology; seafood safety.

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LIST OF ABBREVIATIONS AND SYMBOLS

A

Abs, Absorbance

AChE, Acetylcholinesterase

Acid, Simulated acidification

AIC, Akaike information criterion

ANCOVA, Analysis of co-variance

ANOVA, Analysis of variance

ARSLVT, Administração Regional de Saúde de Lisboa e Vale do Tejo

TA, Total alkalinity

atm, atmosphere

ATPases, Adenosine triphosphate catalystic enzymes

B

B, Bottom area of the tank

BB_{ratio}, Brain to body mass ratio

BCF, Bioconcentration factor

BSA, Bovine serum albumin

bw, Body weight

C

CAT, Catalase

Cd, Cadmium

CDNB, Chloro-2,4-dinitrobenzene

CH_3Hg, Methylmercury

CI, Animal condition index

CO_2, Carbon dioxide

CO_3^{2-}, carbonate ion

CONT feed, Contaminated feed

CTR, Control

Cu, Copper

D

Da, Dalton

DCF, Diclofenac

DDT, Dichlorodiphenyltrichloroethane

Dec, Dechlorane

DGAV, Direcção Geral de Agricultura e Veterinária

DLLME, Dispersive liquid-liquid micro extraction

DNA, Deoxyribonucleic acid

DO, Dissolved oxygen

DoW, Distribution ration

dw, Dry weight

E

EC, European Commission or Enzyme Commission

ECC, Emerging chemical contaminant

EDTA, Ethylenediaminetetraacetic acid

EF, Elimination factor

EFSA, European Food Safety Authority

ELISA, Enzyme-linked immunosorbent assay

ENA, Erythrocyte nuclear abnormality

EPA, Environmental Protection Agency

EPPO, Estação Piloto de Piscicultura de Olhão

Ery, Erythrocytes

Ery:Leu, Erythrocytes:leukocytes ratio

Ery viable, Erythrocytes' viability

EU, European Union

EU/FP7, European Union Seventh Framework Programme

EW, Edible weight

F

FAO, Food and Agriculture Organization

FCT, Fundação para a Ciência e Tecnologia

FCUL, Faculdade de Ciências da Universidade de Lisboa

FEAP, Federation of European Aquaculture Producers

FELASA, Federation of European Laboratory Animal Science Associations

FR, Flame retardant

G

g, grams

$GABA_A$, γ-aminobutyric acid receptor

GC-MS, Chromatography–mass spectrometry

GHG, Greenhouse gas

GLM, General linear models

GLMM, General linear mixed models

GR, Growth rate

GSH, Reduced glutathione

GSSG, Glutathione disulfide

GST, Glutathione S-transferases

H

H, Height

h, hour

HBB, Hexabromobenzene

HCO_3^-, bicarbonate ion

Hg, Mercury

Hg^0, elemental mercury

Hg^{2+}, Mercury cation

HPG axis, Hypothalamo–pituitary–gonadal axis

HPLC, High-performance liquid chromatography

HSC70, Heat shock protein cognate 70 kDa

HSI, Hepatosomatic index

HSP70, Heat shock protein 70 kDa

HSPs, Heat shock proteins

I

iAs, Inorganic arsenic

IBR, Integrated multi-biomarker response

ICNF, Instituto da Conservação da Natureza e das Florestas

IgG, Immunoglobulin G

IPCC, Intergovernmental Panel on Climate Change

IPMA, Instituto Português do Mar e da Atmosfera

IS, Internal standard

IUCN, International Union for Conservation of Nature and Natural Resources

K

K, Fulton's condition index

K^+, Potassium cation

Kg, kilograms

KoW, Octanol-water partition coefficient

L

L, litre

L:D, Light:dark

L_A, Absolute lateralization index

LC-MS/MS, Liquid chromatography-tandem mass spectrometry

Leu, Leukocytes

LOD, Limit of detection

LOEC, Lowest observed effect concentration

Log, Logarithm

LOQ, Limit of quantification

LPO, Lipid peroxidation

L_R, Relative lateralization index

lw, Lipid weight

M

M, Molar concentration

MARE, Marine and Environmental Sciences Centre

MDA, Malondialdehyde bis (dimethylacetal)

MeHg, Methylmercury

mg, milligram

min, minute

mL, mililitre

mm, milimetre

mM, milimolar

MPL, Maximum permissible limit

MS, Mass spectrometry

MSFD, Marine Strategy Framework Directive

MS222, Tricaine methanesulfonate

N

n.a., Not available or not applicable

Na^+, Sodium cation

nano-TiO_2, Titanium dioxide nanoparticles

NAR, Net accumulation rate

NBT, Nitroblue tetrazolium

nd, Not determined or not detected

ng, nanogram

nm, nanometer

nmol, nanomoles

NOAA, National Oceanic and Atmospheric Administration

NOEC, No observed effect concentration

NSAID, Non-steroidal anti-inflammatory drug

O

OC, Organic polutant

P

p, *p*-value

PAHs, Polycyclic aromatic hydrocarbons

Pb, Lead

PBDE, Polybrominated diphenyl ether

PBDEs, Polybrominated biphenylethers

PBS, Phosphate buffered saline

PCB, Polychlorinated biphenyl

pCO_2, Carbon dioxide partial pressure

PFC, Perfluorinated compound

PFOA, Perfluorooctanoic acid

PFOS, Perfluorooctanesulfonic acid

PhAC, Pharmaceutical active compounds

pKa, Ionisation constant

POP, Persistent organic polutant

PPCP, Pharmaceutical and personal care product

ppm, part per million

PTWI, Provisional tolerable weekly intake

PVDF, polyvinylidene fluoride

Q

QuEChERS, Quick easy cheap effective rugged safe

R

r, Pearson correlation coefficient

RAS, Recirculation aquaculture system

RCP, Representative concentration pathways

ROS, Reactive oxygen species

S

s, second

SD, Standard deviation

SDS, Sodium dodecyl sulphate

SNRI, Serotonin-norepinephrine reuptake inhibitor

SOD, Superoxide dismutase

SPE, Solid phase extraction

SST, Sea surface temperature

T

T, Top area of the tank

TBARS, Thiobarbituric acid reactive substances

TBBPA, Tetrabromobisphenol A

TBEP, Tris 2-butoxyethyl phosphate

TCS, Triclosan

Temp, Temperature

T-Hg, Total mercury

TL, Total length

T_n, *n* days of trial

TWI, Tolerable weekly intake

U

U, Units

Ub, Total ubiquitin

UCIBIO, Research Unit on Applied Molecular Biosciences

UK, United Kingdom

UPLC-QqLIT, Ultra-performance liquid chromatography-hybrid quadrupole-linear ion trap

USA, United States of America

UV, Ultraviolet

V

v/v, volume/volume

VFX, Venlafaxine

VTG, Vitellogenin

W

Warm, Simulated warming

WHO, World Health Organization

WI, Width

WS, Water solubility

ww, Wet weight

WWTP, Wastewater treatment plant

X

XOD, xanthine oxidase

Z

Zn, Zinc

SYMBOLS

~, Approximately

[ ], Concentration

˚C, Degree Celsius

<, Lower than

>, Higher than

Δ, Variation

↑ Increase or up-regulation

↓ Decrease or down-regulation

µ, micro

ΩAra, aragonite saturation state

ΩCal, calcite saturation state

CHAPTER 1.

GENERAL INTRODUCTION

Living in a multi-stressors environment

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1.1. Climate change: Causes, trends and projected impacts

The Industrial Revolution constituted a major turning point in History, in which "Man labour" typically used during agrarian times was replaced by "mechanical work", thus, changing forever Man's role in society. Although it represented great progresses in the fields of technology, medicine, economy, education and culture, among others, it also dramatically contributed to the increase of the human footprint on the planet, due to an exhaustive exploitation of natural resources, combustion of fossil fuels, like coal, petroleum (and other oils) and natural gases, and release of pollutants into the environment (IPCC, 2014). Hence, since the late 18th century, human activities have continuously increased the production and emission of greenhouse gases (GHG; e.g. water vapour, carbon dioxide, methane, nitrous oxide and ozone) that absorb and trap solar energy in the form of heat, giving rise to the phenomenon known as "Greenhouse Effect" (**Figure 1.1.**).

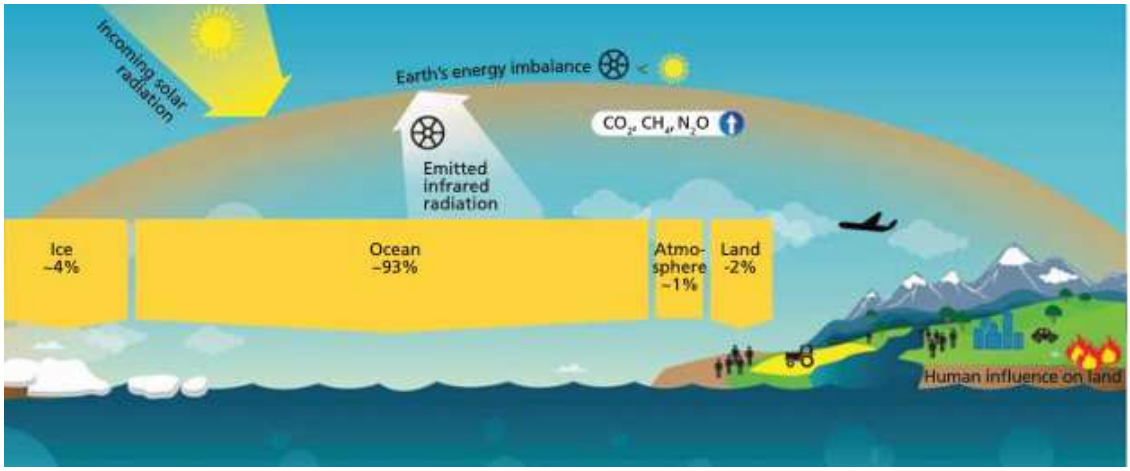

Figure 1.1. Earth's energy budget. Source: Reid (2016).

According to the latest report of the Intergovernmental Panel on Climate Change (IPCC, 2014), GHG emissions have reached unprecedented levels in the last 50 years, unequivocally causing the warming of the planet, with most of the energy produced in the form of heat being stored in the ocean (only ~1% of the total energy produced within the climate system is stored in the atmosphere; Solomon et al., 2007; IPCC, 2014; Reid et al., 2016). Hence, the average seawater surface temperature (SST) has consistently increased about 0.1 ºC per decade since pre-Industrial times (Hansen et al., 2010), and is expected to become warmer, particularly in the Northern Hemisphere (i.e. Northern Atlantic Ocean), reaching up to +5 ºC in temperate coastal areas of the Atlantic ocean by 2100, in an uncontrolled GHG emissions scenario (**Figure 1.2.**; Collins et al., 2013; IPCC, 2014). Within this "global warming" context, a huge set of climate-related effects, which can already be felt in some regions of the planet, are also expected to worsen in a 50-100 year timeframe, including increased seawater stratification, diminished snow cover and rising sea

level with consequent changes in seawater salinity, increased number of "low or dead oxygen zones", as well as alterations of wind and precipitation patterns/intensity and increased frequency, duration (chronicity) and intensity of extreme events, such as heat waves (drastic temperature increases that last, at least, five days in a row), droughts and floods (IPCC, 2014).

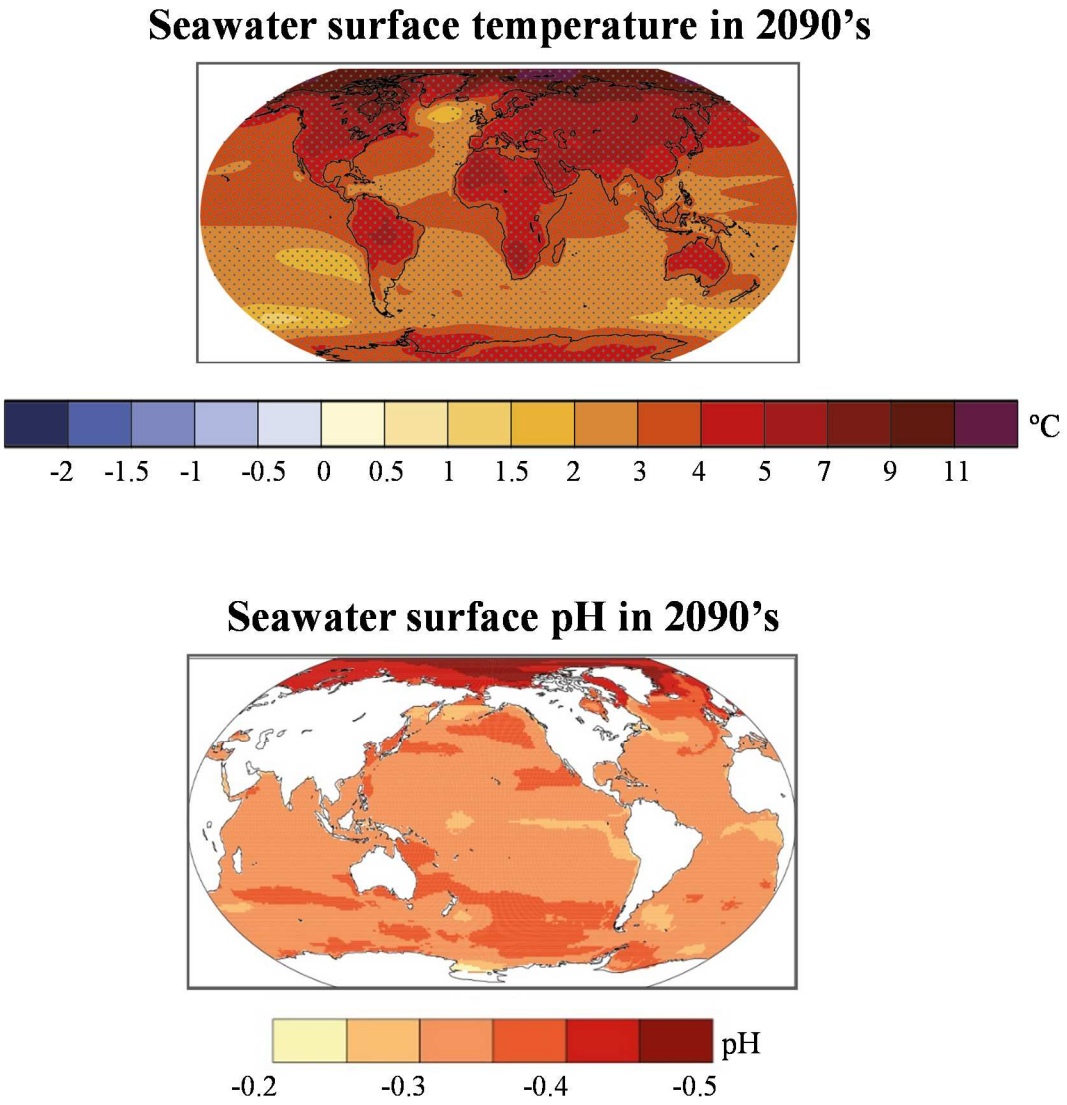

Figure 1.2. Expected seawater surface temperature and pH in the planet by the end of twenty first century (projections according to RCP8.5 of the IPCC Fifth Report Assessment, 2014). Adapted from Ciais et al. (2013) and IPCC (2014).

In addition, the increasing release of GHG has also resulted in a higher oceanic uptake of CO_2 (i.e. increased CO_2 partial pressure, pCO_2), leading to the drop of the average seawater pH at a global scale, i.e. to the so called "ocean acidification" (IPCC, 2014; McNeil and Sasse, 2016). In fact, despite having exhibited relatively stable values for more than 800 million years, it has been recently estimated that the average seawater surface pH has dropped approximately 0.1 units in

relation to pre-industrial times, therefore, representing a 26% increase in ocean acidity (IPCC, 2014). Globally, acidification rate is estimated to be ~50% faster in Northern regions than in subtropical and tropical ones (FAO, 2018a). At a global scale, and even if efforts are made to keep GHG emissions at today's rates, recent projections indicate that, by the end of this century, seawater pCO_2 levels will still rise up to +1000 µatm, corresponding to a drop of ~0.4 pH units, in temperate coastal areas of the Atlantic ocean (**Figure 1.2.**; Ciais et al., 2013; IPCC 2014; McNeil and Sasse, 2016).

Depending on the region, each of the above mentioned climate change effect can occur in isolation or, in worst-cases, combined with other climate-related or non-climate-related stressors (e.g. pollution), promoting a cascade sequence of events that can result in an overall exacerbation of the impacts (IPCC, 2014; FAO, 2018a). For instance, both warming and salinity (or both) can promote water stratification, due to either the enhanced formation of pycnoclines (i.e. layer in a water mass with higher density, due to salinity and temperature increase), or to lower surface salinity (e.g. from increased freshwater run-off; e.g. Diaz and Rosenberg, 2008). On the other hand, increased stratification may result in lower oxygen diffusion from the upper part of the water column to the lower part, translating into an increased number of hypoxic events or dead zones in bottom waters (Breitburg et al., 2018).

Regardless of acting alone or combined, climate change effects will certainly have an impact on marine biota, affecting their fitness, metabolism, reproduction, recruitment and distribution, among other ecological features (Rosa et al., 2014, 2016; FAO, 2018a), raising the need for species to adapt to the new prevailing environmental conditions or, in most extreme cases, leading to their extinction. Marine species inhabiting estuaries and coastal areas are thought to be particularly sensible to climate change impacts, since these shallow water ecosystems usually have a weaker hydrodynamic activity (compared to the "open ocean") and, thus, naturally exhibit pronounced monthly or even daily shifts of abiotic conditions due to the influence of season and/or tides, among other factors (Barbosa, 2010; Madeira et al., 2015, 2016; Rodrigues et al., 2017; FAO, 2018a). In addition, since estuaries and coastal areas not only are shelter ecosystems to many bivalve, crustacean and fish species during their early and most vulnerable life stages (i.e. they are important spawning and nursery areas), but also house many aquaculture facilities, climate change effects are expected to strongly affect these areas, likely causing biodiversity reduction and economic losses associated with the fisheries and aquaculture sectors. On the other hand, new opportunities may also emerge from climate change, such as the exploitation of new wild/farmed marine resources.

Seafood constitutes an important source of protein and essential nutrients (vitamins, minerals and omega-3 fatty acid), and is one of the most globally traded food commodities, assuring the livelihood of millions of people (FAO, 2018a). Yet, by constituting an additional challenge to the ecological success of marine species, climate change effects can certainly have a negative impact

in the fisheries sector, by altering the primary production, biodiversity (Barbosa, 2010), species' distribution and population structure of marine ecosystems (Barbosa, 2010; FAO, 2018a). In addition, climate change effects, particularly warming, are also foreseen to increase the incidence and severity of pathogens (e.g. bacteria, virus, parasites, toxins from harmful algal blooms) and to exacerbate water pollution (Marcogliese, 2008; Noyes et al., 2009; Marques et al., 2010; Rosa et al., 2012; Manciocco et al., 2014). Such risks can not only compromise the resilience of seafood species, but also rise many seafood safety concerns. Changes in fisheries productivity are already visible worldwide and will become even more evident in the coming 100 years. For instance, reaching a drop of the total catches up to 15% in Portugal's mainland (and slightly higher losses in the Portuguese islands of Madeira and Azores), according to the scenario RCP8.5 of the IPCC (IPCC, 2014; FAO, 2018a). Over the last years, aquaculture has been significantly contributing to meet the global demand for seafood products, enabling a stronger nutritional and socio-economic development of many countries. Despite this sector has experienced a slower growth rate since the 1990's, aquaculture is expected to increase its contribution to the global seafood production in the future, in order to continue fulfilling the nutritional needs of a growing world population (FAO, 2018a). Still, due to climate change, major challenges will be also expected for aquaculture, mostly as a result of: i) the reduced availability of wild seeds; ii) species' altered thresholds of physiological tolerance (e.g. altered thermal window), which may force the replacement of formerly cultured species by new and more resilient ones (Rosa et al., 2012; Madeira et al., 2015); iii) increased occurrence and incidence of pathogens, harmfull algal blooms and parasites, and exacerbation of water pollution (particularly, in extensive and semi-intensive rearing systems), therefore, compromising seafood safety (Marcogliese, 2008; Noyes et al., 2009; Marques et al., 2010; Rosa et al., 2012; Manciocco et al., 2014); and iv) limitation of water resources in regions, associated with reduced precipitation or increased number of drought and heat-wave events (Rosa et al., 2012; FAO, 2018a).

1.2. Emerging chemical contaminants (ECCs)

In the marine environment, abiotic variations (including climate change-related ones) are not the only stressors that compromise the resilience of marine organisms. In fact, chemical contamination constitutes another consequence of the increasing "human footprint" on the planet, being one of the greatest environmental concerns from our time. Marine ecosystems, especially those close to highly urbanized areas, such as estuaries and coastal lagoons, are known to be particularly prone to chemical contamination (e.g. Bollman et al., 2012; Maulvault et al., 2015), as they constitute the ultimate destination for several pollutants that are carried over through riverine and atmospheric processes, or directly discharged via domestic, industrial, agricultural and hospital effluents (Huerta et al., 2012; Santos et al., 2013). Once in the environment, chemical

contaminants can persist for many years and be uptaken by marine biota, promoting multiple adverse effects, not only to these species but also to humans through the ingestion of contaminated seafood (Marques et al., 2010).

Several efforts were made to assure the sustainability of the marine environment and its natural resources. In 2008, the European Commission (EC) released the Marine Strategy Framework Directive (MSFD), which constituted the first piece of legislation in the field of marine environmental policy and a formal commitment of the Member States to reduce or cease marine polycyclic aromatic hydrocarbons (PAHs), polybrominated biphenylethers (PBDEs) and toxic metals (i.e. mercury, Hg, cadmium, Cd, and lead, Pb; Directive 2008/56; EC, 2008). From the human health perspective, and at the European level, maximum permissible limits (MPLs) for the occurrence of some chemical contaminants in seafood products were also established (Directive 1881/2006; EC, 2006a).

Yet, in addition to the well-known chemical contaminants mentioned above, and whose presence in the environment (and seafood species) is already regulated and regularly monitored, new chemical substances not historically recognized as pollutants are also increasingly more present in the marine environment (e.g. Feo et al., 2012; Cunha et al., 2015; Marques et al., 2015; Vandermeersch et al., 2015). These "emerging chemical contaminants" (ECCs) comprise a wide variety of substances with distinct physical and chemical properties and modes of actions that share the lack of regulation and the urgent need for a deep understanding of the risks that they may pose to the environment and humans. Substances defined as ECCs are mostly man-made (e.g. flame retardants, FRs, perfluorinated compounds, PFCs, and pharmaceutical and personal care products, PPCPs), though some may also occur naturally (e.g. inorganic arsenic, iAs, and methylmercury, MeHg; e.g. Bollman et al., 2012; Feo et al., 2012; Maulvault et al., 2015; Vandermeersch et al., 2015).

In light of the limited scientific knowledge, in 2015, the EC has created the so called “Watch List” of non-regulated ECCs for which further monitoring and toxicological data is urgently needed, in order to accurately estimate their ecological risks and decide, in the future, whether or not they should be included in the MSFD, as well as in seafood regulations (EC Decision 2015/495; EC, 2015). Despite the “Watch List” constitutes a great progress towards the regulation of ECCs in the environment, so far, it only includes ten hazardous substances (most of them belonging to the PPCPs group), thus, leaving out a wide number of compounds for which the ecotoxicological impacts are still unknown.

Table 1.1. presents a brief description of the ECCs addressed in this PhD thesis, including their physical and chemical properties, occurrence in aquatic biota and toxicity (whenever available).

Table 1.1. Summary of ECCs' physical-chemical properties and toxicity.

Compound	Methylmercury (MeHg)	Inorganic arsenic (iAs, i.e. sum of AsIII or AsV)	Diclofenac (DCF)	Venlafaxine (VFX)	Triclosan (TCS)
ECC category	Toxic metal species	Toxic metal species	PPCP	PPCP	PPCP
Main commercial use	None (formerly used in pesticide formulation)	None (formerly used in pesticide formulation)	Non-steroidal anti-inflammatory drug (human and veterinary use)	Antidepressant drug (human use)	Antibacterial and antimicotic compound (human use)
Sources	Natural and synthetic	Natural and synthetic	Synthetic	Synthetic	Synthetic
Occurrence in aquatic biota	up to 20 mg kg^{-1} ww in predatory fish	up to 50 mg kg^{-1} ww in bivalves and macroalgae	up to 9 ng g^{-1} dw in fish	up to 36 ng g^{-1} dw in bivalves	up to 507 ng g^{-1} dw in fish
Molecular formula	CH_3Hg^+	As^{3+} or As^{5+} species (e.g. H_3AsO_4)	$C_{14}H_{11}Cl_2NO_2$	$C_{17}H_{27}NO_2$	$C_{12}H_7Cl_3O_2$
Molecular weight	215.6 g mol^{-1}	Varies according to species (e.g. H_3AsO_4: 185.9 g mol^{-1})	296.1 g mol^{-1}	277.4 g mol^{-1}	289.5 g mol^{-1}
Water Solubility (WS at 20 ºC)	100 mg L^{-1}	Varies according to species (e.g. for H_3AsO_4, WS = 5900 g L^{-1}	4.82 mg L^{-1}	572 g L^{-1}	1.2 g L^{-1}
Biotransformation products	demethylated in Hg^{2+}	As^{5+} reduced to As^{3+}	4'-Hidroxydiclofenac, 5'-Hidroxydiclofenac and diclofenac glucuronide	O-desmethylvenlafaxine, N-desmethylvenlafaxine, NO-didesmethylvenlafaxine, NN-didesmethyl-O-desmethylvenlfaxine	Glucuronide- and sulfate-TCS conjugates
pKa	na	Varies according to species (e.g. H_3AsO_4: between 2.2 and 13.4)	4.1	9.6	8.1
Log KoW	0.62	na	4.51	3.20	4.76
Estimated half-life in humans	~80 days	~4 days	~2 hours	~5 hours	~21 hours
Toxicity in marine biota	NOEC: 1.5-4.0 µg L^{-1}	NOEC: 0.5 µg L^{-1}; LOEC: 1.0 µg L^{-1}	NOEC: 320-1000 µg L^{-1}	LOEC: 31.3 µg L^{-1}	NOEC: 34.1 mg L^{-1}; LOEC: 71.3 mg L^{-1}

Table 1.1. ***(continuation)*** Summary of ECCs' physical-chemical properties and toxicity. Abbreviations: ECC, emerging chemical contaminant; PPCP, pharmaceutical and personal care product; FR, flame retardant; PFC, perfluorinated compound; na, not available; NOEC, no observed effect concentration; LOEC, lowest observed effect concentration; Log KoW, logarithm of the octanol-water partition coefficient; pKa, acid dissociation constant; dw, dry weight; ww, wet weight; lw, lipid weight.

Compound	Dechlorane 602 (Dec 602)	Dechlorane 603 (Dec 603)	Dechlorane 604 (Dec 604)	Tetrabromobisphenol A (TBBPA)	Perfluorooctanesulfonic acid (PFOS)	Perfluorooctanoic acid (PFOA)
ECC category	FR	FR	FR	FR	PFC	PFC
Main commercial use	Production of fibreglass-reinforced nylon	na	Production of commercial greases	Production of fire-resistant polycarbonates	Production of stain-resistant carpets, textiles, electronic devices, photolithographic film, fire-fighting foams, and surfactants	Production of polytetrafluoroethylene (PTFE, i.e. Teflon), as well as fire-fighting foams, wetting agents and cleaners
Sources	Synthetic	Synthetic	Synthetic	Synthetic	Synthetic	Synthetic
Occurrence in aquatic biota	up to 34 ng g^{-1} lw in fish	up to 0.55 ng g^{-1} lw in fish	up to 1.3 ng g^{-1} lw in fish	up to 245 ng g^{-1} lw in fish	500 ng g^{-1} ww in predatory fish	between 1.8 and 2.4 ng g^{-1} ww in fish
Molecular formula	$C_{14}H_4Cl_{12}O$	$C_{17}H_8Cl_{12}$	$C_{13}H_4Br_4C_6$	$C_{15}H_{12}Br_4O_2$	$C_8HF_{17}O_3S$	$C_8HF_{15}O_2$
Molecular weight	613.6 g mol^{-1}	637.7 g mol^{-1}	692.5 g mol^{-1}	543.9 g mol^{-1}	500.1 g mol^{-1}	414.1 g mol^{-1}
Water Solubility (WS at 20 ºC)	8.49 ng L^{-1}	0.3 ng L^{-1}	2.21 ng L^{-1}	0.063 mg L^{-1}	0.52 mg L^{-1}	3.4 mg L^{-1}
Biotransformation products	na	na	na	Conjugated metabolites (i.e. sulfate-, glucuronide- and mixed glucuronidesulfate-TBBPA conjugates)	na	na
pKa	na	na	na	7.5	< 1.0	1.3
Log KoW	7.10	8.50	8.50	5.90	na (not measurable)	na (not measurable)
Estimated half-life in humans	na	na	na	~13 hours	~5 years	~4 years
Toxicity in marine biota	na	na	na	NOEC: 0.017 mg L^{-1}; LOEC: 0.032 mg L^{-1}	NOEC: 0.3 mg L^{-1}; LOEC: 0.6 mg L^{-1}	NOEC: 24 mg L^{-1}

References used in this Table: US National Research Council (2000); Orvos et al. (2002); Scheytt et al. (2005); Sandborgh-Englund et al. (2006); Schauer et al. (2006); EFSA (2008); Singh et al. (2008); Giesi et al. (2010); Feo et al. (2012); Huerta et al. (2013); Memmert et al. (2013); Álvarez-Muñoz et al. (2015); The Danish Environmental Protection Agency (2015); Dhillon et al. (2015); Fong et al. (2015); Gutu et al. (2015); Jo et al. (2015); Vandermeersch et al. (2015); Lee et al. (2017); DrugBank (2018); Pittinger and Pecquet (2018); PubChem (2018a,b,c).

1.2.1. Toxic metals (MeHg and iAs)

Toxic elements, such as Hg, Cd, Pb and arsenic (As) are elements with no known biological function in living organisms that can occur in marine ecosystems due to both natural or anthropogenic causes. They have been long known to bioaccumulate in marine biota (e.g. Maulvault et al., 2015), causing several adverse effects in these species, as well as in seafood consumers (EFSA, 2009a,b, 2010, 2012a). Although toxic elements can be present in different molecular forms (e.g. Hg can be found in its elemental, Hg^0, inorganic, Hg^{2+} and Hg_2^{2+}, or organic forms, e.g. CH_3Hg), or even alternate between forms according to the surrounding abiotic conditions, for many years elemental speciation in the environment was not thoroughly monitored nor it is still considered in the current regulations regarding the presence of contaminants in seafood (regulation No 1881/2006; EC, 2006a). Yet, assessing elemental speciation is highly relevant from an ecological and food safety point of view, as some toxic elements, like Hg and As, are known to predominantly occur in their most toxic forms in seafood species (Muñoz et al., 2000; Maulvault et al., 2015). Hence, a risk assessment not taking into account the distinct contributions of each molecular form to the total toxic element burden can lead to unrealistic estimations of the health risks related to toxic elements' dietary exposure (e.g. Maulvault et al., 2014).

Methylmercury is the most toxic Hg species and the predominant one in marine organisms, particularly in predatory fish species with long life cycles (Afonso et al., 2006; Maulvault et al., 2011, 2015). In contrast, the most toxic As forms are the inorganic ones (iAs, i.e. the sum of all AsIII and AsV molecular forms) that predominate in seawater and marine sediments (Francesconi and Edmonds, 1996), often reaching high concentrations in bivalve and macroalgae species (see **Table 1.1.;** Maulvault et al., 2015). So far, only a few studies investigated the speciation of these elements in seafood species (e.g. Maulvault et al., 2015), and the assessment of human dietary exposure to MeHg or iAs has been mostly based on extrapolations from the total element contents (i.e. total Hg and total As) found in food matrices (e.g. EFSA, 2009b, 2012a). For this reason, worldwide, food safety authorities have recently adverted that further research and monitoring programs should be undertaken in order to increase the available data on the presence of MeHg and iAs in all food groups to enable a more accurate and reliable exposure risk assessment (e.g. EFSA, 2009b, 2012a).

Regarding the toxicity of these elements to aquatic biota, a recent study on MeHg toxicity reported no observed effect concentrations (NOECs) of 1.5 µg L^{-1} and 4.0 µg L^{-1} in rotifer *Brachionus koreanus* and copepod *Paracyclopina nanafor* species, respectively (Lee et al., 2017). It should be noted, though, that since the majority of the studies performed so far only assessed the acute and chronic toxicity of MeHg water exposure, NOECs for dietary exposure are still unavailable, despite MeHg is mostly uptaken through dietary sources and is subsequently

transferred along the food chain (Miniero et al., 2013). As for iAs, Gutu et al. (2015) reported a NOEC of 0.5 µg L^{-1}, as well as a lowest observed effect concentration (LOEC) of 1.0 µg L^{-1} in *Artemia salina*.

1.2.2. Pharmaceuticals and personal care products (PPCPs)

Pharmaceuticals and personal care products comprise a wide diversity of compounds, including human and veterinary pharmaceuticals, cosmetics, preservatives, detergents, among others. Their presence in marine ecosystems has become a great environmental concern in the last years, as PPCPs are often discharged into rivers, estuaries and seas, due to their rather limited elimination during conventional wastewater treatments (Huerta et al., 2012). Domestic, hospital and cosmetic industry effluents, as well as agriculture and aquaculture activities are the main sources of PPCPs contamination in coastal environments (Santos et al., 2013). These compounds are considered pseudo-persistent contaminants, as the rate at which they are introduced in the aquatic environment often exceeds their rate of degradation.

Diclofenac (DFC), carbamazepine, citalopram, venlafaxine (VFX), diazepam, sotalol and sulphamethoxazole are some of the pharmaceutical active compounds (PhACs) most commonly found in the marine environment (e.g. Beretta et al., 2014; Vandermeersch et al., 2015; Rodriguez-Mozaz et al., 2017). On the other hand, triclosan (TCS) and methylparaben are two compounds often used as additives of various personal care products, therefore, also being frequently detected in seawater and marine biota (e.g. Vandermeersch et al., 2015). Despite the occurrence of PPCPs in seafood is still poorly monitored, recent data revealed that these compounds can bioaccumulate in marine organisms (Huerta et al., 2012; Vandermeersch et al., 2015; Serra-Compte et al., 2018), promoting several adverse effects at the biochemical, cellular and behavioural levels (e.g. Bisesi Jr. et al., 2014; Gonzalez-Rey and Bebianno, 2014; Rowett et al., 2016). NOECs and LOECs for DCF, VFX and TCS, which are target compounds in this PhD thesis, are presented in **Table 1.1.** Furthermore, there is a growing body of evidence that some PPCPs (e.g. TCS and DCF) can also interfere with the neuroendocrine system of marine biota (e.g. Gonzalez-Rey and Bebianno, 2014), as well as of mammals (e.g. Feng et al., 2016) and, thus, they are also classified as endocrine disrupting compounds.

Although the presence of PPCPs in both the environment and seafood is still not regulated in the EU, some compounds (e.g. DCF, 17α-ethynylestradiol, 17β-estradiol) have been recently included in the "Watch List" of emerging non-regulated aquatic pollutants. Yet, recent data suggests that, apart from the 33 substances already prioritized by the MSFD (2008/56/EC) and the 10 substances placed under the EC "Watch List" (EC Decision 2015/495) there is a huge set of PPCPs that could potentially be classified as "priority substances" and, perhaps, regulated in the future (Álvarez-Muñoz et al., 2015; Vandermeersch et al., 2015).

1.2.3. Flame retardants (FRs)

Flame retardants are mixtures of man-made chemicals added to several industrial and household products (e.g. plastics, textiles, and electrical/electronic equipment) to make them less flammable. This group includes various compounds from different categories, including polybrominated diphenyl ethers (PBDEs), chlorinated substances (Mirex, dechloranes), tetrabromobisphenol A (TBBPA), hexabromobenzene (HBB), and organophosphorous compounds (e.g. tris 2-butoxyethyl phosphate – TBEP). Since most FRs are extremely hydrophobic, these substances can be highly persistent in the environment, particularly accumulating in marine sediments and biota (e.g. Álvarez-Muñoz et al., 2015; Vandermeersch et al., 2015). FRs' concentrations in seawater are usually bellow detection or within the pg L^{-1} range (Bollmann et al., 2012; Satín et al., 2013; Poma et al., 2014), whereas in marine sediments and biota their concentrations are in the ng g^{-1} and µg g^{-1} ranges (Satín et al., 2013; Poma et al., 2014).

TBBPA is one of the most relevant and widely used FRs, being frequently found in river and estuarine sediments, and reaching concentrations up to 245 ng g^{-1} lipid weight (lw) in marine biota (EFSA, 2011; Vandermeersch et al., 2015). Although distinct toxicological effects have been observed in marine organisms acutely or chronically exposed to different TBBPA concentrations, the most sensitive endpoint was attributed to blue mussel *Mytilus edulis* shell growth following 70 days of exposure (i.e. estimated NOEC: 0.017 mg L^{-1}; estimated LOEC: 0.032 mg L^{-1}; Pittinger and Pecquet, 2018).

After the Stockholm Convention, some compounds intensively used in the past (e.g. PBDEs, Mirex) were banned or restricted in the EU (Council Decision 2006/507/EC; EC, 2006b), being subsequently replaced by new substances, like HBB, TBEP and dechloranes (Decs 602, 603 and 604; Feo et al., 2012) for which the hazards to the environmental and human health are still unknown. Even though data regarding the environmental occurrence of these "emerging FRs" is extremely limited, recent studies have reported Decs' total concentrations up to 3.7 ng g^{-1} dw in sediments and up to 27.0 ng g^{-1} lw in biota (Giulivo et al., 2017). Based on the limited available data, particularly in what concerns "emerging FRs", in 2009, the European Food Safety Authority (EFSA) has drawn a recommendation to further monitor the presence of these contaminants in food items, including seafood (EFSA, 2012b).

1.2.4. Perfluorinated compounds (PFCs)

Perfluorinated compounds (e.g. perfluorooctanesulfonic acid, PFOS, and perfluorooctanoic acid, PFOA) are molecules composed by carbon chains strongly bound to fluorine atoms, which are widely used in various industrial and consumer activities (e.g. stain-resistant coatings for fabrics and carpets, fire-fighting foams and floor polishes, among others). Their strong

carbon:fluorine bounds make them extremely resistant to degradation and, therefore, persistent over time in biological compartments. In aquatic environments, PFCs concentrations can vary from non-detectable values up to 9 ng L^{-1} in coastal areas, and reach over 70 ng L^{-1} in wastewater treatment plants (WWTPs; Flores et al., 2013). Despite variations according to species and location (i.e. between polluted or clean areas), PFCs concentrations can range from 0.1 ng g^{-1} in molluscs to over 500 ng g^{-1} in predatory fish species and, therefore, seafood has been pointed out as an important pathway for human exposure to PFCs (EFSA, 2008; Vandermeersch et al., 2015).

In what concerns toxicological data, PFOS's NOEC and LOEC in aquatic organisms have been set at 0.30 mg L^{-1} and 0.60 mg L^{-1}, respectively, whereas lower toxicity has been attributed to PFOA (values estimated for fathead minnow, *Pimephales promelas*; Giesi et al., 2010). Based on their frequent detection in seafood species, EFSA published a Scientific Opinion of the Panel on Contaminants in the Food Chain stating that there is a clear need to improve the dataset to enable the accurate assessment of the potential risks associated with the human exposure to PFCs (EFSA, 2008).

1.3. Linking climate change and ECCs: What do we know so far?

Chemical contaminants' speciation, transfer among biological compartments and bioavailability in marine sediments/water column are strongly influenced by environmental drivers, such as temperature, pH, salinity, upwelling and stratification events (e.g. Noyes et al., 2009; Marques et al., 2010). On the other hand, abiotic conditions also play a key role on marine organisms' physiology and ecological success (e.g. Rosa et al., 2014, 2016; Madeira et al., 2015, 2016), conditioning the way these species cope with the simultaneous occurrence of other environmental stressors, such as chemical contamination. Thus, by altering species physiological status and, at the same time, exacerbating many forms of water pollution, climate change effects can potentially hinder marine species to cope with the presence of chemical contaminants in the same way as they did before (e.g. Marques et al., 2010; Freitas et al., 2016; Rowett et al., 2016; Sampaio et al., 2016), therefore, rising several concerns from both the ecological and seafood safety perspectives. Yet, understanding the potential interactions between climate change-related stressors and pollution is a topic that only recently raised attention within the scientific community and, therefore, further research efforts, particularly focusing on the non-regulated and poorly studied ECCs, are urgently required to better forecast the ecotoxicological implications of climate change.

The following sub-chapters provide an overview of the state of the art regarding the potential interactions between climate change effects and ECCs' bioavailability in the marine environment (sub-chapter *1.3.1*) and toxicity to marine biota (sub-chapter *1.3.2*).

1.3.1. ECCs' fate and bioavailability in tomorrow's ocean

Starting with organic compounds (OC), including pesticides, polychlorinated biphenyl (PCBs), FRs, PFCs, dioxins, polycyclic aromatic hydrocarbons (PAHs), among others, temperature is one of the most relevant factors influencing their distribution, half–life in biological compartments, volatilisation, re-emission (Gouin and Wania, 2007; Teran et al., 2012), and altering their partitioning into the different phases (solid, liquid and gas) (Macdonald et al., 2002). For instance, increased temperatures can enhance OCs' volatilisation and, consequently, enhance exchanges between the ocean and atmosphere, which can result in reductions in contaminant exposure to marine biota (Armitage et al., 2011; Nadal et al., 2015). Organic pollutants that evaporate can form contaminated gases in temperate and tropical areas, being subsequently transported to higher latitudes in hop events, i.e. in quick jumps that occur seasonally in the course of natural climate fluctuations. In such cases, less pronounced temperature gradients between different regions of the globe may remarkably alter OCs' atmospheric partitioning, affecting their fate and distribution (Beyer et al., 2002). On the other hand, warming along with increased precipitation, can also exacerbate contaminant degradation, translating into a diminished volatilization into the atmosphere and, consequently, reduced transport of the contaminant to higher latitudes (Dalla Valle et al., 2007; Nadal et al., 2015).

In a cascade sequence of events, increased snow melting and the consequent sea level rising that are closely linked to global warming may facilitate OCs' exchanges between the air and water compartments (Macdonald et al., 2005). Depending on the geographic area, both projected climate change outcomes will directly influence salinity levels in aquatic systems. On one hand, seawater level rise due to climate change can promote the intrusion of saltwater in previous freshwater environments, particularly in subtropical areas, estuaries and coastal ecosystems (IPCC, 2014). Since OC's are less soluble in saltwater, enhanced bioavailability and toxicity can be expected in these areas (Noyes et al., 2009). Conversely, in other regions of the planet, increased precipitation and snow/ice melting will result in higher input of freshwater into the ocean promoting lower salinity levels (IPCC, 2014) and leading to opposite effects in OCs' bioavailability.

Toxic metals (e.g. Hg, Pb, Cd and As) solubility and speciation is largely dependent on seawater temperature and pH and, as such, environmental variations associated to climate change will certainly have preponderant effects on metals' availability and concentration in the marine environment. This will alter metals' behaviour and transfer from sediments into the water column and vice-versa, as well as their toxicity (Marques et al., 2010). Additionally, since metal inputs into aquatic systems are strongly linked to climate events, such as snow melting and precipitation, alterations in elemental profiles, distribution and concentrations are likely expected due to climate change (Marques et al., 2010; Hoffmann et al., 2012). As discussed above for OCs, if the climate

continues changing, great alterations of salinity regimes will occur due to sea level rise, increased snow melting and occurrence of extreme events, like storms, floods and droughts. Metals' ability to precipitate, bind or release from sediments are largely determined by environmental characteristics, such as pH, cation exchange capacity, organic matter content, redox conditions and chloride content. Therefore, varying salinities can likely affect metal mobility in intertidal sediments, influencing bioavailability and toxicity to biota, as demonstrated in previous studies (Hatje et al, 2003; Du Laing et al., 2002, 2008). For instance, Du Laing et al. (2007) reported a reduction of metal accumulation in sediments of intertidal reed beds along the Scheldt river with increased salinities. The same author also assessed the effect of flood water on heavy metals' mobility in intertidal sediments (Scheldt estuary, Belgium), revealing that higher salinity levels enhanced metal mobility, particularly Cd (Du Laing et al., 2008). Following changes in seawater temperature and salinity, which can lead to stratification, low dissolved oxygen levels (hypoxia) can also facilitate the release of metals from sediments to the water column (Schiedek et al., 2007). Apart from changes in bioavailability and mobility, increased seawater temperatures can also have a prevalent role in elements' speciation and, consequently, in their toxicity to biota (Marques et al., 2010; Hoffmann et al., 2012). For instance, warmer temperatures, as well as reduced oxygen levels (or even anoxia) facilitate Hg methylation by bacteria, thus increasing the uptake of this element along the food chain in its most toxic and persistent form, i.e. MeHg (Booth and Zeller, 2005).

Until now, PPCPs are by far one of the less studied groups of ECCs, especially within a climate change context. Despite being relatively new pollutants, for which the available data is still limited, recent studies suggest that environmental conditions play a key role in the chemical behaviour, degradation and metabolization of this type of pollutants, emphasizing the need to further assess the potential effects of climate change when investigating the toxicological risks of PPCPs (e.g. Azzouz and Ballesteros, 2013). Since most substances are extremely sensitive to light, heat and surrounding pH conditions (Moreno et al., 2009; Welankiwar et al., 2013; Gul et al., 2015), the expected warming, increased UV radiation due to a depleted ozone layer, and acidification can likely exacerbate PPCPs' degradation in the aquatic environment, depending on the stability degree of each compound, likely enhancing their toxicity to biota (Macdonald et al., 2005; Schiedek, 2007; Azzouz and Ballesteros, 2013).

Finally, when establishing possible interactions between climate change and the fate, distribution and bioavailability of some particular compounds, namely PPCPs and pesticides, various indirect effects also need to be taken into account. As previously mentioned, the projected changes in climate, such as warming, which will subsequently alter species distribution and result in diminished physiological condition of other species, will also have a preponderant role on the occurrence, distribution, toxicity and virulence of pathogenic organisms, including parasites, bacteria, virus and microalgae (Dale et al., 2006; Marcogliese, 2008; Donavaro et al., 2011). Thus,

the growing incidence and broader distribution of diseases and plagues, which affect humans and animals (particularly farmed), will certainly promote utilization of pharmaceuticals and plague controlling products, leading to enhanced environmental contamination, especially in coastal marine ecosystems close to highly urbanized areas or in the vicinities of farming facilities (Marcogliese, 2008; De Silva and Soto, 2009; IPCC, 2014). This clearly raises the need not only to better understand the ecotoxicological implications of increased PPCPs exposure under climate change context, but also to assess animal immune responses to diseases.

1.3.2. Coping with ECCs in tomorrow's ocean: carry-over and toxicity to aquatic biota

Environmental variables not only directly influence chemical contaminants' availability and environmental distribution, but also indirectly alter compounds' toxicological aspects (Noyes et al., 2009). In this sense, when assessing the effects of climate change, from an ecotoxicological point of view, two aspects should be taken into account: i) bioaccumulation and ii) toxicity.

In aquatic animals, the bioaccumulation of chemical contaminants occurs primarily at the respiratory and gastrointestinal epithelia, which are tissues biologically designed for a rapid and efficient uptake. Once uptaken, chemical contaminants are then distributed to different target organs via blood, alone or in association with transporter proteins. In order to deal with the presence of toxicants, a series of biological mechanisms takes place, involving compound biotransformation and detoxification through the enzymatic machinery (e.g. cytochrome P450, GST), followed by its subsequent excretion (Tierney et al., 2014). Among other ecological factors (e.g. origin, age, nutritional status), the ability of an organism to cope with the presence of contaminants and to detoxify them is largely dependent on its fitness, physiological status and metabolic rates which, then again, are greatly influenced by the surrounding environmental conditions (**Figure 1.3.**; Dijkstra et al., 2013; Freitas et al., 2016; Anacleto et al., 2018).

Because adapting to one set of environmental stressors implies great physiologic efforts and, therefore, increased susceptibility to other existing stressors, the interactions between climate change and chemical contaminants can be looked from two different angles. On one side, by affecting biota's metabolism, climate changes can result in altered bioaccumulation/detoxification mechanisms and toxicological responses to contaminant exposure (e.g. Noyes et al., 2009; Marques et al., 2010; Dijkstra et al., 2013; Anacleto et al., 2018). On the other side, organisms already living on the edge of their physiological capacities due to constant inputs of pollutants will certainly exhibit lower tolerance to environmental changes and will struggle to adapt to the new prevailing conditions (e.g. Noyes et al., 2009; Marques et al., 2010; Manciocco et al., 2014).

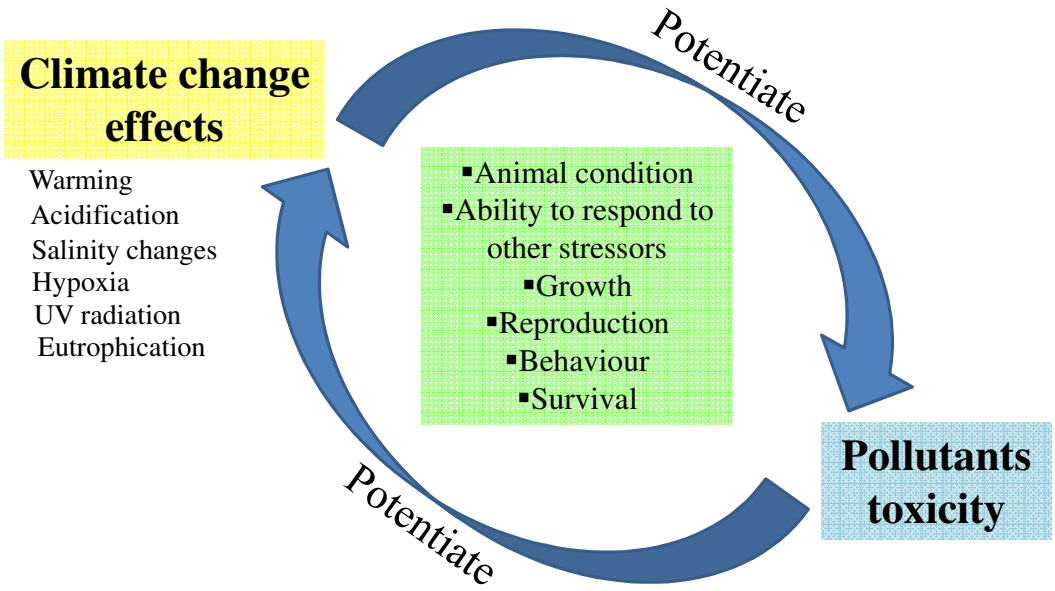

Figure 1.3. Interaction between climate change effects and chemical contaminants.

In this context, the following sections present an overview of the available data, gathered from recent field and laboratory studies concerning the ecotoxicological responses of marine species subjected to the combination of climate change effects and pollution. Despite a wide variety of environmental factors, acting alone or combined with other stressors, would also worth attention, the impacts of ocean warming and acidification on contaminants' toxicity will be addressed in these sections due to the prevalence of these variables in the current state of the art with respect to climate change effects on marine ecosystems, as well as the aims of the present PhD thesis (i.e. to focus on the effects interactive effects of ocean warming and acidification).

Ocean warming

Out of the environmental variables affected by climate change, to date, temperature is one of the best documented parameter from a marine and ecological point of view (e.g. Madeira et al., 2015, 2016; Anacleto et al., 2018). Since most marine species are ectothermic, temperature is a crucial variable to their physiological functioning. Though many organisms have evolved to cope with daily or seasonal temperature variations, when multiple environmental stressors take place concurrently, species resilience to temperature peak events or drastic seasonal changes may be surpassed, thus compromising its survival (e.g. Madeira et al., 2012). In addition, despite genetic adaptation may enable some species or populations to persist and overcome natural selection pressures induced by changing environmental temperatures, such adaptations can also lead to reduced genetic diversity and, therefore, from an evolutionary perspective, this could be looked at as a downside (Jager et al., 2016). Noteworthy, the physiological stress and genetic adaptation promoted by such changes are expected to have even more pronounced ecological consequences

in tropical species, which are already living near their thermal tolerance threshold, thus, being generally more susceptible to temperature variations than their temperate counterparts (Rosa et al., 2016). Such is the case of tropical coral reefs that typically live in symbiosis with dinoflagellates. Because temperature increase promotes the release of these organisms from corals, seawater warming for extended periods may compromise the stability of such symbiotic interactions, resulting in coral bleaching, diminished growth, increased susceptibility to pollutants and diseases, mortality and, ultimately, higher probability of extinction (Hoegh-Guldberg et al., 2007).

In marine biota, both reproduction and stress response mechanisms are regulated by the endocrine system, together with the nervous system. Thus, in the presence of endocrine disrupting pollutants (e.g. triclosan, 17α-ethynylestradiol, 17β-estradiol, phthalates, and PCBs), deleterious effects have been reported in marine organisms including, among others, the induction or depression of vitellogenin-like protein synthesis (i.e. egg-yolk precursors) (Matozzo et al., 2012; Dias et al., 2014), abnormal embryonic development (Hwang et al., 2014), impairment of the thyroid endocrine system (Zhai et al., 2014), loss of reflex action (Hedrick-Hopper, et al., 2015) and increased stress-hormone (e.g. cortisol) levels (Teles et al., 2016). The last two responses being closely linked to increased animal stress. On the other hand, because spawning events are, in many cases, strongly influenced by temperature, seawater warming is expected to have a preponderant effect on species' fecundity timing and success (Przeslawski et al., 2008). Thus, taking into consideration the direct effects of endocrine disrupting pollutants and seawater temperature acting in isolation, it can then be hypothesized that synergetic interactions may take place if these two environmental stressors occur simultaneously. Yet, given the current lack of empirical and consistent data supporting this hypothesis (Brown et al., 2015; Hedrick-Hopper et al., 2015), whether global warming can potentiate or not the deleterious impacts of endocrine disrupting pollutants on species reproduction and stress response is a matter that deserves to be further investigated.

When it comes to ocean warming, the metabolic changes induced by thermal stress are one of the most studied physiological responses in marine biota (e.g. Neuheimer et al., 2011; Holt and Jørgensen, 2015; Madeira et al., 2016; Anacleto et al., 2018). In general, organisms subjected to warmer temperatures exhibit enhanced metabolism, accompanied by increased ventilation and feeding rates in response to higher metabolic demands. Such changes can translate into higher contaminant bioaccumulation (contaminants dissolved in the water column, i.e. via respiration, or present in feeds or natural preys, i.e. via ingestion) and elimination rates (i.e. contaminant metabolization and excretion) (e.g. Dijkstra et al., 2013; Sampaio et al., 2016). Yet, to adequately assess the relation between warming and contaminants' bioaccumulation/elimination patterns, animal growth efficiency is a parameter that deserves careful consideration when interpreting data

because: i) an enhanced metabolism also implies higher energetic costs to biota, often leading to lower animal condition and fitness (Johnston and Dunn, 1987; Stauber et al., 2016); and ii) animal feeding rates cannot be seen as directly proportional to its metabolic demands, since the amount of ingested food is largely determined not only by prey/feed availability, but also by predator behaviour (Dijkstra et al., 2013). In addition to changes in contaminant bioaccumulation patterns, alterations in animal metabolism and condition induced by warming can also exacerbate compound toxicity, lowering the ability of an organism to successfully respond to contaminants and/or to detoxify them, or increasing their biotransformation into more toxic compounds (e.g. Marques et al., 2010; Manciocco et al., 2014; Stauber et al., 2016).

Finally, it should be further stressed that, given the previously described ambivalence in climate change and contaminant interactions, with the first potentiating impacts of the second or *vice-versa*, the sole exposure to contaminants can also influence an organism's thermal tolerance, i.e. species critical thermal maximum (CTmax; Stauber et al., 2016).

Ocean acidification

Ocean acidification has the potential to directly or indirectly influence marine species physiology, welfare and survival (Rosa et al., 2017). At a first glance, the effects of ocean acidification seem to be more noticeable and dramatic to calcified organisms, to which the development and growth are intrinsically dependent on the equilibrium of the calcium carbonate cycle. For this reason, over the years, great research efforts have been channelled towards the assessment of potential ecological threats of ocean acidification to marine invertebrates, where most studies have been focused on coral reefs, followed by bivalves and crustaceans (e.g. Kleypas et al., 2006; Hoegh-Guldberg et al., 2007; Cohen and Holcomb, 2009). Nevertheless, despite fish species are known to be relatively tolerant to pH variations, since they are capable of adjusting their internal pH according to the surrounding levels (Fabry et al., 2008), recent studies have revealed that hypercapnia can lead to the development of body malformations, as well as changes in buoyancy and loss of spatial orientation (Gutowska et al., 2010; Pimentel et al., 2014).

As previously discussed, contaminants' chemical properties are largely influenced by environmental conditions, with metals and other ionic compounds being particularly affected by the surrounding seawater pH levels. Such is the case of the PPCP TCS that becomes increasingly protonated and loses its negative charge as pH decreases, which, on the other hand, translates into enhanced compound availability and toxicity to biota (Orvos et al., 2002; Rowett et al., 2016). Indeed, the recent study of Rowett et al. (2016), using the freshwater amphipod *Gammarus pulex* as model organism, evidenced increased TCS toxicity under lower pH levels. As argued by these authors, such trend is explained by the fact that lipid membranes are generally impermeable to ionised molecular forms, and that TCS requires a pH value around 8.0 pH units to become ionized

(i.e. in its less toxic form; Lyndall et al., 2010; Rowett et al., 2016). Similarly, two recent studies using marine bivalves (i.e. peppery furrow shell clam *Scrobicularia plana,* Freitas et al., 2016; Japanese carpet shell clam *Ruditapes philippinarum,* Munari et al., 2016), also reported synergistic interactions between the surrounding pH level and the PPCPs carbamazepine and diclofenac, with specimens exhibiting higher mortality and oxidative stress when exposed to the combination of acidification plus PPCPs, than those exposed to each stressor in isolation. Another study performed on the immune system of the Korean mussel *Mytilus coruscus* exposed to low pH conditions and titanium dioxide nano-particles (nano-TiO_2), which are new substances with a growing interest in ecotoxicology, revealed interactive and carry-over effects, i.e. reduction of total haemocyte counts, phagocytosis, esterase, and lysosomal content, as well as, increased haemocyte mortality and formation of reactive oxygen species (ROS; Huang et al., 2016).

When addressing the toxicological impacts of acidification, it is also worthwhile to consider its effects on biota's behaviour. Such effects are mostly attributed to the fact that hypercapnia disrupts the ionic balance in proton-based neurotransmitter receptors, such as the γ-aminobutyric acid receptor ($GABA_A$) neurotransmitter, translating into increased animal anxiety and boldness (Hamilton et al., 2014; Munday et al., 2014; Sampaio et al., 2016; Lai et al., 2017).

Furthermore, recent studies have evidenced that, given the high conservation of the nervous system throughout evolution within vertebrates, marine pollutants that are neurotoxic to humans (e.g. MeHg) or were designed to modulate specific human behaviours (i.e. psycho-active drugs, such as antidepressants, anxiolytics, anticonvulsants), may also promote similar responses in fish (Valenti et al., 2012; Brooks, 2014; Fong and Ford, 2014; Sampaio et al., 2016). Despite the recent findings consistently pointing out ocean acidification to potentially promote neurotoxicological aspects of these contaminants (Bisesi Jr. et al., 2014; Sampaio et al., 2016), further research on this topic should be carried out to accurately explore different fish behavioural cues, neurological functioning and the ecotoxicological impacts of climate change.

Finally, as described in the recent review of Nikinmaa (2013) and considering the ambivalence of such interactions, it should be highlighted that pollutants by themselves can also indirectly contribute to ocean acidification, by affecting animal abundance and survival in three ways: i) high levels of chemical contaminants (including ionic metals, that are essential at some levels, but can become toxic at high concentrations) compromise the primary production, by reducing the abundance of photosynthetic organisms in relation to the number of heterotrophic organisms, thereby, disrupting the CO_2 equilibrium in the ocean (i.e. the ratio between what is produced and what is consumed); ii) the elevated mortality of calcified organisms caused by the exposure to pollutants can induce calcium carbonate sinking to the bottom of the ocean, impairing the natural carbon cycle (e.g. Cohen and Holcomb, 2009); and iii) seawater eutrophication can lead to increased aerobic respiration rates (i.e. enhanced CO_2 production), but the presence of pollutants

may decrease photosynthesis inducing an overall acidification in some areas of the planet (Nikinmaa, 2013).

1.4. Thesis aims, experimental approach and layout

1.4.1. Research questions and experimental approach

The increased human footprint on the planet since the beginning of the Industrial Revolution has resulted in two of the greatest environmental concerns of our time: remarkable chemical contamination and climate change. Both environmental stressors strongly threaten the resilience of marine ecosystems and are expected to worsen in the future, compromising both marine species' ecological success and seafood safety. Yet, as this research topic only started to raise attention among the scientific community in the last years, information regarding the potential interactions between climate change and chemical contamination is still extremely limited, particularly in what concerns non-regulated ECCs for which even less toxicological data is currently available. Hence, this calls for the urgent need to research and gather a sufficient amount of data enlightening the most appropriate directions for future environmental management, ECCs' regulation and mitigation of climate change impacts in an integrated and multi-disciplinary way. Within this context, the present PhD thesis aimed to provide an important contribution to this innovative and poorly studied research area, by particularly addressing the interactive effects of warming and acidification on ECCs' bioaccumulation and ecotoxicological responses in marine invertebrate and vertebrate species. Hence, this PhD dissertation poses the following three specific research questions:

1. Will warming and/or acidification affect ECCs' bioaccumulation and elimination mechanisms in marine biota?

2. If so, will seafood consumers be at a greater risk in tomorrow's ocean?

3. Will warming and/or acidification affect marine species' ecotoxicological responses to ECCs?

To address these questions, thirteen non-regulated ECCs from different chemical groups and with distinct modes of action were selected, including toxic element species (MeHg and iAs), PPCPs (DCF, VFX and TCS), FRs (Decs 602, 603 and 604 and TBBPA) and PFCs (PFOS and PFOA). Although ECCs can be uptaken by marine biota through water (via inhalation) and diet (throughout the food chain) and, sometimes, one exposure pathway can predominate over the other one depending on compound's chemical behaviour, dietary exposure was prioritized in this PhD thesis (i.e. only two trials tackled ECCs water exposure; see **Chapters 4.** and **6.**), since studies assessing ECCs exposure through trophic transfer are currently extremely limited.

Regarding the experimental approach for the exposure to climate change-related stressors, as described in following chapters (**Chapters 2.-6.**), seawater warming and/or acidification were simulated, in all cases, according to the most recent projections of the Intergovernmental Panel

for Climate Change (IPCC) for the worst-case scenario of climate change effects, i.e. scenario RCP8.5 (ΔTemperature = +4-5 ºC and ΔpCO_2 = ~+1000 µatm, equivalent to ΔpH = -0.4 units; IPCC, 2014; McNeil and Sasse, 2016). A full cross-factorial experimental setup was carried out in all trials, in order to explore all potential interactive effects between variables/stressors.

Four independent trials (i.e. one per each studied compound) were carried out for MeHg, DCF, VFX and TCS using juvenile fish species (i.e. European seabass, *Dicentrarchus labrax* for MeHg and DCF, meagre *Argyrosomus regius* for VFX and white seabream *Diplodus sargus* for TCS) as biological models (the choice of fish species was based on the criteria described in sub-Chapter *1.4.2.*, as well as in species' availability at the time of the trial). In these four trials, both compound bioaccumulation/elimination and toxicity to juvenile fish were investigated (see **Parts 1.** and **2.** of **Chapters 2.-5.**). The first trial performed within this PhD program was focused solely on the effect of warming on MeHg bioaccumulation/elimination and toxicity (see **Chapter 2., Parts 1.** and **2.**), and constituted a pilot study that allowed to optimize the incorporation of ECCs in commercial fish feeds, as well as the functioning of the recirculation aquaculture system (RAS) used to simulate climate change effects. Once these key points were optimized and adjusted to the experimental needs, seawater acidification was also introduced as a variable, i.e. warming and acidification were simulated, acting alone or combined, in all subsequent trials (see **Chapters 3.-5.**).

Finally, with the specific purpose of complying with one of the main objectives of the FP7 project ECsafeSEAFOOD (supporter of the work performed during this PhD program), a fifth trial simulating both warming and acidification was also carried out, this time focusing on relevant ECCs mixtures (including iAs, Dec 602, Dec 603 Dec 604, TBBPA, PFOS and PFOA) and using commercial marine bivalve species (*Mytilus galloprovincialis* and *Ruditapes philippinarum*) as biological models (see **Chapter 6.**). Considering the logistic limitations (i.e. the number of available tanks in the RAS used in these trials) which did not allow to study the effect of these ECCs acting alone (i.e. in a non-mixture context), this trial was only devoted to the assessment of ECCs' bioaccumulation/elimination mechanisms.

1.4.2. Marine fish and bivalves species as biological models to assess the interactive effects of climate change and ECCs

To address to three main research questions of this PhD thesis, three juvenile marine fish (*D. labrax*, *A. regius* and *D. sargus*) and two bivalves species (*M. galloprovincialis* and *R. philippinarum*) were selected as biological models. **Table 1.2.** presents a summary of the ecological features, optimal rearing conditions and commercial importance of the selected biological models. Such selection was based on the following criteria:

1. Species typically inhabiting coastal areas (e.g. estuaries and coastal lagoons) because, as previously described (**Chapter 1.1.**), these ecosystems (and the species inhabiting them) can be particularly sensible to climate change impacts. Even though marine biota inhabiting these unstable environments have developed different ecological strategies to cope with great daily or monthly amplitudes of abiotic conditions, recent evidence suggested that these species are extremely vulnerable to climate change effects, as they already live close to their physiological thresholds and have limited acclimation plasticity (e.g. Madeira et al., 2012, 2015). On the other hand, the conservation, biodiversity and economic value of estuaries and coastal lagoons is also greatly compromised by the presence of chemical contaminants, since these ecosystems are often located near highly urbanized and industrialized areas and, therefore, are constantly exposed to discharges of domestic, agricultural and industrial effluents (Barbosa, 2010; Maulvault et al., 2015; Rodrigues et al., 2017).

2. Species likely to bioaccumulate high levels of ECCs, such as predatory fish that are particularly susceptible to ECCs dietary exposure (trophic transfer) through the ingestion of contaminated preys, and bivalve species that are sedentary and benthic filter-feeding organisms, thus being susceptible to ECCs present in both the water column and contaminated sediments (Álvarez-Muñoz et al., 2015; Maulvault et al., 2015; Vandermeersch et al., 2015).

3. Species with high commercial value, to link bioaccumulation data with the potential impacts of climate change in seafood safety, as well as in the fisheries and aquaculture sectors.

4. Specifically for fish species, specimens at the juvenile stage were preferred because early life stage organisms often exhibit higher sensitivity to environmental stressors than adult ones. In addition, changes in the fitness and ecotoxicological responses of juvenile organisms can potentially affect species' recruitment and overall ecological success, thus negatively impacting the fisheries and aquaculture sectors.

Table 1.2. Summary of the ecological features and commercial importance of the marine fish and bivalve species selected as biological models of this PhD thesis.

	Species	*Dicenthrarchus labrax*	*Argyrosomus regius*	*Diplodus sargus*	*Mytilus galloprovincialis*	*Ruditapes philippinarum*
						
Common name		European seabass	Meagre	White seabream	Mediterranean mussel	Japanese carpet shell clam
Geographical distribution		Northern Atlantic coast, Mediterranean Sea and Black Sea	Northeastern Atlantic coast, Mediterranean Sea and Black Sea	Eastern Atlantic coast, Mediterranean Sea and Black Sea	Mediterranean Sea, Eastern Atlantic coast, Eastern Pacific coast,	Indo-Pacific, Northern American Pacific coast, Northern Atlantic coast and Mediterranean sea
Habitat		Coastal waters, estuaries and brackish waters and rivers	Estuaries and rocky coastal waters	Coastal waters, estuaries and brackish waters	Intertidal zone, estuaries and rocky shores	intertidal zone, brackish waters and estuaries
Optimal rearing conditions (main parameters)	**Temperature**	5 ºC-28 ºC (~20 ºC preferred)	13 ºC-28 ºC (~20 ºC preferred)	14 ºC-28 ºC (~19 ºC preferred)	10 ºC-20 ºC (~19 ºC preferred)	15 ºC-28 ºC (~19 ºC preferred)
	pH	~8.0 pH units	~8.0 pH units	~8.0 pH units	~8.0 pH units	~8.0 pH units
	Salinity	3‰-39‰	5‰-39‰	5‰-39‰	~34‰	24‰-35‰
Feeding habits		Predator (juveniles and adults): smaller fish, crustaceans and molluscs	Predator (juveniles and adults): smaller fish, crustaceans and molluscs	Omnivorous (juveniles and adults): seaweeds and benthic invertebrates	Filter-feeder	Filter-feeder
Reproduction	**Biological strategy**	Gonochoristic; Batch spawner	Gonochoristic; Batch spawner	Gonochoristic or sequencial hermaphrodite (protandric); Batch spawner	Gonochoristic; Broadcast spawner	Sequential hermaphrodite; Broadcast spawner
	Maturation age	~3 years	> 4 years	~2 years	~1 year	~1 year
	Spawning period	Winter-early Summer	Late Spring-Summer	Winter-early Spring	Late Autumn-early Spring	Spring-early Summer
Commercial interest	**Global capture production (2014)**	8,401 tons	5,000-10,000 tons	3,378 tons	1,080 tons	33,170 tons
	Aquaculture production (2014)	156,449 tons	11,770 tons	13 tons	116,262 tons	4,010,702 tons
	Price	High	High	High	Low (compared to other bivalves, e.g. *R. philippinarum*)	Low (compared to other bivalves, e.g. *R. decussatus*)
Conservation (IUCN red list)		Least concern	Least concern	Least concern	Not evaluated	Not evaluated

References used in this Table: Kaschner et al., 2010; Okaniwa et al., 2010; Dülger et al., 2012; Lee et al., 2012; Fountoulaki et al., 2017; FAO, 2018b,c,d,e,f; IUCN, 2018.

1.4.3. Thesis layout

This thesis comprises eight chapters, with the first chapter presenting an overview of the state of the art regarding climate change and ECCs sources and impacts, as well as the potential interactive effects between these stressors. The information gathered during the conception of **Chapter 1.** allowed to prepare a book chapter entitled *"Chemical Contaminants in a Changing Ocean"* that will be included in the book *"Ecotoxicology of Marine Organisms"* edited by Science Publishers (see **Annex 1.**). **Chapters 2.-6.** are devoted to each of the target ECCs of this PhD thesis, i.e.:

Chapter 2. – The biomagnifying and potent neurotoxicant methylmercury (MeHg). This chapter is divided in **Part 1.**, dedicated to compound bioaccumulation and elimination in *D. labrax* subjected to warming, and **Part 2.**, dedicated to the *D. labrax* ecotoxicological responses (i.e. animal fitness indexes, antioxidant and heat shock responses, and neurotoxicity). Each part led to one separate published manuscript (see **Annex 1.**).

Chapter 3. – The non-steroidal anti-inflammatory drug diclofenac (DCF). This chapter is also divided in two parts, with **Part 1.** presenting an integrated multi-biomarker approach (including animal fitness indexes, haematological parameters, antioxidant responses, protein chaperoning and degradation, endocrine disruption and neurotoxicity) to assess the interactive effects of warming, acidification and DCF exposure on *D. labrax* ecotoxicological responses (corresponding to the third published manuscript; see **Annex 1.**). Due to limitations (and weaknesses) of the experimental design, as well as of the analytical methodology used to quantify DCF, this compound was not detected in any tissue samples collected throughout this trial, therefore, not allowing the preparation of a manuscript devoted to DCF bioaccumulation and elimination. Still, it was decided to include a second part in this chapter in order to discuss the potential causes for such unexpected absence of positive results (see **Part 2.**).

Chapter 4. – The antidepressant drug venlafaxine (VFX). This chapter is structured in a similar way to the previous ones (i.e. **Part 1**. VFX bioaccumulation and elimination using *A, regius*, i.e. published manuscript 4, and **Part 2.** *A. regius* ecotoxicological responses, integrating fish antioxidant responses, protein chaperoning and degradation, endocrine disruption and neurotoxicity, i.e. published manuscript 5; see **Annex 1.**). Yet, it also includes a third part devoted to fish behaviour (i.e. published manuscript 6), given the psychotropic mode of action of this particular compound (see **Part 3.**). In addition, considering the fact that pharmaceutical active drugs are assumed to be mostly uptaken by marine biota via water and, thus, studies concerning other possible compound exposure pathways are extremely limited, differences between water

and feed exposure routes (in terms of bioaccumulation, ecotoxicological and behavioural effects) are also discussed throughout this chapter.

Chapter 5. – The antimicrobial and anti-mycotic triclosan (TCS), previously associated with endocrine disruption in both biota and humans. In this chapter, both compound bioaccumulation (**Part 1.**) and ecotoxicological responses in *D. sargus* (including animal fitness indexes, antioxidant responses, protein chaperoning and degradation, endocrine disruption and neurotoxicity; **Part 2.**) are presented, with these two parts being merged in a single manuscript devoted to TCS (published manuscript 7; see **Annex 1.**).

Chapter 6. – ECCs mixtures, including inorganic arsenic (iAs), dechloranes (Dec 602, Dec 603 and Dec 604), tetrabromobisphenol A (TBBPA), perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA). As previously mentioned, due to logistic limitations, it was not possible to assess the ecotoxicological responses of ECCs mixtures. Hence, this chapter is only dedicated to the effects of climate change on ECCs' bioaccumulation and elimination mechanisms in marine bivalves (*M. galloprovincialis* and *R. philippinarum*), following water or dietary exposure, according to the chemical behaviour of each compound (i.e. published manuscript 8; see **Annex 1.**). Yet, the relevance of further addressing this subject is later discussed in **Chapter 8**.

Then, in **Chapter 7. General Discussion** the research questions initially raised in this PhD thesis (**Chapter 1.4.1.**) are clearly addressed, by discussing the results presented in **Chapters 2.-6.** in an integrated way.

Finally, the main take-home messages, as well as a critical analysis of the methodologies and results obtained during the preparation of this PhD thesis are presented in **Chapter 8.**, "leaving the door opened" for future research topics that could contribute to a deeper and wider understanding of the impacts of climate change, as well as to the sustainable management of marine ecosystems in tomorrow's ocean.

1.5. References

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CHAPTER 2.

TOXIC METALS: THE PERSISTENT, BIOMAGNIFYING AND NEUROTOXIC METHYLMERCURY AS A CASE STUDY

Part 1.

Bioaccumulation and elimination of mercury in juvenile seabass (*Dicentrarchus labrax*) in a warmer environment

Manuscript 1.

Maulvault, A.L., Custódio, C., Anacleto, P., Repolho, T., Pousão, P., Nunes, M.L., Diniz, M., Rosa, R., Marques, A., 2016. Bioaccumulation and elimination of mercury in juvenile seabass (*Dicentrarchus labrax*) in a warmer environment. *Environmental Research*, 149, 77–85. DOI: 10.1016/j.envres.2016.04.035.

Abstract

Warming is an expected impact of climate change that will affect coastal areas in the future. These areas are also subjected to strong anthropogenic pressures leading to chemical contamination. Yet, the consequences of both factors for marine ecosystems, biota and consumers are still unknown. The present work aims to investigate, for the first time, the effect of temperature increase on bioaccumulation and elimination of mercury [(total mercury (T-Hg) and methylmercury (MeHg)] in three tissues (muscle, liver, and brain) of a commercially important seafood species – European seabass (*Dicentrarchus labrax*). Fish were exposed to the ambient temperature currently used in seabass rearing (18 °C) and to the expected ocean warming (+4 °C, i.e. 22 °C), as well as dietary MeHg during 28 days, followed by a depuration period of 28 days fed with a control diet. In both temperature exposures, higher MeHg contents were observed in the brain, followed by the muscle and liver. Liver registered the highest elimination percentages (EF; up to 64% in the liver, 20% in the brain, and 3% in the muscle). Overall, the results clearly indicate that a warming environment promotes MeHg bioaccumulation in all tissues (e.g. highest levels in brain: 8.1 mg kg^{-1} ww at 22 °C against 6.2 mg kg^{-1} ww at 18 °C after 28 days of MeHg exposure) and hampers MeHg elimination (e.g. liver EF decreases after 28 days of depuration: from 64.2% at 18 °C to 50.3% at 22 °C). These findings suggest that seafood safety may be compromised in a warming context, particularly for seafood species with contaminant concentrations close to the current regulatory levels. Hence, results point out the need to strengthen research in this area and to revise and/or adapt the current recommendations regarding human exposure to chemical contaminants through seafood consumption, in order to integrate the expected effects of climate change.

Keywords: Seawater warming, European seabass, Methylmercury, Bioaccumulation, Elimination.

1. Introduction

Climate change and expected impacts on coastal ecosystems is a widely debated theme, raising several environment, animal welfare and human health related concerns. Despite this research topic is still taking its first steps, and uncertainties are often raised when projecting possible causes and consequences of climate change, by the end of the 21st century, it is expected, with a high degree of confidence, the increase in frequency and severity of extreme temperature events (heat waves) (IPCC, 2014). Seawater temperature has increased during the last three decades, particularly in the Northern hemisphere, and is expected to become warmer, increasing the global mean sea surface temperature (SST) between 2.6 to 4.8 ºC, in a 50 to 100 years' timeframe (IPCC, 2014). Coastal areas, lagoons and estuaries will be particularly affected by climate change since they are subjected to a wide range of environmental drivers and anthropogenic activities (e.g. industrial, agricultural and domestic pollution), likely negatively affecting the performance and survival of marine biota inhabiting these vulnerable ecosystems (Rosa et al., 2014) and promoting alterations at molecular, physiological and behavioural levels (e.g. Anacleto et al., 2014a,b; Pimentel et al., 2014).

The enhancement of chemical contaminants toxicity to marine organisms and its potential negative effects on seafood safety is also a noteworthy aspect of ocean warming. Indeed, alterations of hydrographical conditions such as seawater temperature increase may, on one hand, directly influence contaminants bioavailability in the water column and sediments (e.g. facilitate the methylation of Hg in aquatic systems since the methylation rates are temperature dependent; Downs et al., 1998), and, on the other hand, affect the metabolism of biota, thus, likely affecting contaminants uptake/elimination rates (e.g. Marques et al., 2010; Dijkstra et al., 2013; Rosa et al., 2014; Manciocco et al., 2014). Since seafood plays an important role in the human diet at a global scale, and especially to coastal populations, possible changes in contaminants bioaccumulation/elimination mechanisms in these species may imply great health related concerns. So far, limited studies investigated the way marine organisms will cope with chemical contaminants in a climate change context, and even fewer assessed seafood safety aspects in the oceans of tomorrow (Marques et al., 2010).

European seabass (*Dicentrarchus labrax*) is a commercially important fishery resource and one of the most relevant farmed fish species in Europe (reaching over 70,000 tonnes in 2012) (FEAP, 2013; FAO, 2014). As top-predatory species, wild juveniles feed on small invertebrates (shrimps and benthic organisms, such as bivalves, gastropods and polichaetes), whereas adults are mostly piscivorous (FAO, 2015), thus likely accumulating higher levels of biomagnifying chemical contaminants, such as methylmercury (MeHg) (e.g. Miniero et al., 2013). Furthermore, by inhabiting areas particularly vulnerable to hydrographic alterations (i.e. coastal waters, estuaries and river mouths in the wild, or often reared in offshore aquaculture systems), climate

change effects may certainly represent great ecological and toxicological challenges to this fish species (Marques et al., 2010; Rosa et al., 2012).

In this context, the aim of this study is to investigate the effect of environmental warming on Hg accumulation and elimination in European seabass (*Dicentrarchus labrax*) juveniles. Special emphasis was put on the most toxic and predominant Hg form in seafood species, i.e. MeHg, for which maximum permissible concentrations (MPC's) have not yet been established.

2. Materials and methods

2.1. Control and MeHg-enriched diets

Non-contaminated (control) and contaminated dry inert pellets (MeHg-enriched) with the same nutritional composition were manufactured by a specialized feed producing company (SPAROS Lda, Olhão, Portugal), considering the nutritional requirements for juvenile seabass (detailed feed composition can be consulted in **Annex 2, Table A.2.1.**). Ingredients were ground (below 250 µm) in a micropulverizer hammer mill (model SH1, Hosokawa-Alpine, Germany). Powdered ingredients were then mixed according to the target formulation in a double-helix mixer (model 500L, TGC Extrusion, France). No oils were incorporated at this stage. Pellets (size: 3.0 mm) were produced in a twin-screw extruder (model BC45, Clextral, France) with a screw diameter of 55.5 mm and temperature ranging 105-109 °C, followed by drying in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). After cooling, oils were added by vacuum coating to the extruded pellets (model PG-10VCLAB, Dinnisen, The Netherlands). For the MeHg-enriched diet, MeHg(II) chloride (CH_3ClHg, 99.8%, Sigma-Aldrich) was dissolved in a small volume of ethanol (< 6 mL, 100% v/v) and then mixed with the oils before pellets extrusion. Diets were maintained at 4 °C, and MeHg stability in the diets was evaluated throughout the experiment. Methylmercury concentration in control and MeHg-enriched diets were, in dry weight (dw), 0.60 ± 0.01 mg kg^{-1} and 8.02 ± 0.14 mg kg^{-1} (mean ± standard deviation, n = 3; see also **Annex 2, Table A.2.2.**), respectively. Both selected concentrations were considered to be ecologically relevant and representative of the low [~0.12 mg kg^{-1} wet weight (ww)] and high (~1.6 mg kg^{-1} ww) levels of Hg contamination encountered in species inhabiting contaminated coastal areas, susceptible to accumulate Hg, and that are natural preys of juvenile seabass (e.g. Cardoso et al., 2014). Proximate chemical composition of the diets was determined according to the following procedures: dry matter by drying at 105 °C for 24 h; ash by combustion at 550 °C for 12 h; crude protein by a flash combustion technique, followed by a gas chromatographic separation and thermal conductivity detection (nitrogen analyser FP428, LECO, USA), using a nitrogen conversion factor of 6.25; lipid content was determined by the Soxhlet method, as described by the Association of Official Analytical Chemists (AOAC, 2005).

2.2. Organisms and acclimation

European seabass (*Dicentrarchus labrax*) with similar biometric characteristics were reared until juvenile stage (12.8 ± 0.7 cm total length; 19.2 ± 4.0 g total weight; **Table 2.1.1**) at the aquaculture pilot station of the Portuguese Institute of the Atmosphere and the Sea (IPMA, Olhão, Portugal) using routine hatchery conditions. Afterwards, organisms were transported to the aquaculture facilities of Laboratório Marítimo da Guia (Cascais, Portugal), where they were randomly and equitably distributed in 12 rectangular shaped glass tanks (100 L), each with independent recirculating systems. Each system was equipped with independent temperature (Frimar, Fernando Ribeiro Lda, Portugal) and pH control (adjusted automatically with a Profilux controlling system connected to individual pH probes that monitor the pH values every 2 s; Kaiserslautern, Germany), protein skimmer (Reef SkimPro, TMC Iberia, Portugal), UV sterilization (Vecton 300, TMC Iberia, Portugal), biological filtration (FSBF 1500, TMC Iberia, Portugal) and chemical filtration (active charcoal). To avoid physiological stress, fish load was kept below 5 g body weight L^{-1} in each tank. On a daily basis, dead fish and faeces were removed and 10% of the water volume was exchanged in each incubation tank. Ammonia (NH_3/NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) concentrations were daily checked (Tropic Marin, USA), and kept below detectable levels (i.e. $NH_3/NH_4^+ < 0.25$ mg L^{-1} and $NO_2^- < 0.10$ mg L^{-1}), with the exception of nitrates, which were kept below 2.0 mg L^{-1}. Specimens were acclimated for a period of 30 days and kept under the following conditions: dissolved oxygen > 5 mg L^{-1}; temperature = 18 ± 0.5 °C; pH = 8.00 ± 0.05, salinity = 35 ± 1 ‰ and photoperiod of 12 hours light and 12 hours dark (12L:12D). Five days before initiating the exposure phase, seawater temperature was slowly raised (1 ºC per day) until reaching 22 ± 0.5 ºC in tanks simulating seawater warming conditions (i.e. treatments 22_control and 22_MeHg-enriched; description of treatments is provided in **section 2.3.**), to allow specimens to acclimate to this temperature. Fish were fed with 2% of the average animal body weight at least three times a day with the control diet, i.e. total feed provided was daily adjusted accounting both mortalities and biometric variations throughout time in each tank. Mortality during acclimation was below 1%.

2.3. MeHg exposure/elimination experiment

Four treatments were carried out, each comprising three tanks or replicates (n = 20 animals per tank; **Figure 2.1.1.**): treatment 1: 18_control (control conditions), i.e. seawater temperature set at 18 °C (i.e. average seawater temperature currently used in juvenile seabass rearing in Iberian Peninsula) and animals fed with the control diet; treatment 2: 18_MeHg-enriched, i.e. seawater temperature set at 18 °C and animals fed with the MeHg-enriched diet; treatment 3: 22_control, i.e. seawater temperature set at 22 °C (i.e. simulating seawater warming, Δ = 4 °C according to

IPCC projections; IPCC, 2014) and animals fed with the control diet; treatment 4: 22_MeHg-enriched, i.e. seawater temperature set at 22 °C and animals fed with the MeHg-enriched diet. During the exposure phase, juvenile seabass specimens were fed with the respective diets (control or MeHg-enriched) during 28 days, following the procedure described in **section 2.2**. Afterwards, an elimination phase was carried out for 28 days by daily feeding animals from all treatments with the control diet. Seawater physical-chemical parameters were daily checked and adjusted at optimum levels whenever needed as previously described (**section 2.2.**). No mortality was observed during the experimental trial. Six animals were randomly sampled from each treatment (two fish per tank/replicate) on days 0, 7, 14, 28, 35, 42 and 56. Fish trials were approved by the Ethical Committee of the Faculty of Sciences of the Lisbon University and conducted according to legal regulations (EU Directive 2010/63). Fish were euthanized by cervical sectioning and dissected to remove brain, liver and muscle tissues. Fish biometric data were recorded (**Table 2.1.1.**), and tissue samples were immediately frozen at -80 °C and freeze-dried at −50 °C, 10^{-1} atm of vacuum pressure for 48 h (Power Dry LL3000, Heto, Czech Republic), homogenized and kept at -80 °C until further analysis. Water samples (n = 3) were also collected from each tank in the sampling day and kept at 4 °C until the analysis of mercury levels.

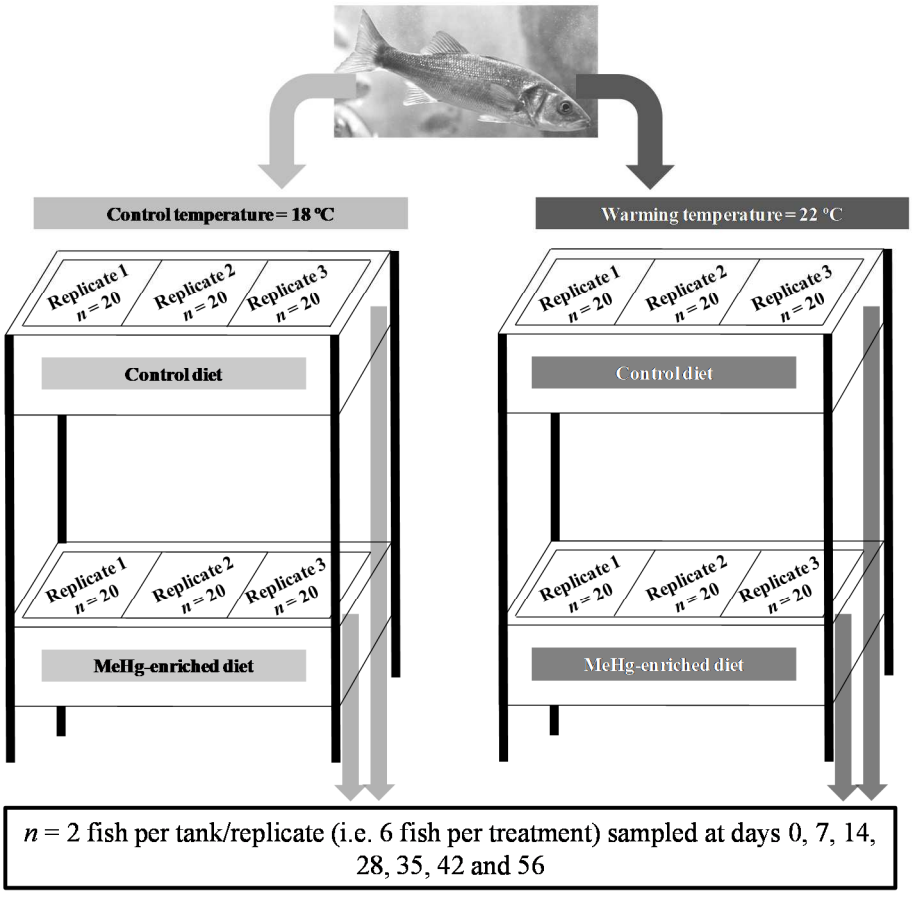

Figure 2.1.1. Experimental design scheme.

Table 2.1.1. MeHg content in non-contaminated fish (mean ± standard deviation; mg kg^{-1}), as well as total length (mean ± standard deviation, cm) and weight (mean ± standard deviation, g) of specimens from all treatments (n = 6). In each column, different letters indicate significant differences between sampling days ($p < 0.05$; $df = 15$). Abbreviations: 18_control – seawater temperature set at 18 °C and animals fed with control diet; 18_MeHg-enriched – seawater temperature set at 18 °C and animals fed with MeHg-enriched diet; 22_control – seawater temperature set at 22 °C and animals fed with control diet; 22_MeHg-enriched – seawater temperature set at 22 °C and animals fed with MeHg-enriched diet.

	MeHg content (mg kg^{-1})						Length				Weight			
	18_control			22_control			18_control	18_MeHg-enriched	22_control	22_MeHg-enriched	18_control	18_MeHg-enriched	22_control	22_MeHg-enriched
	Muscle	Liver	Brain	Muscle	Liver	Brain								
Day 0	0.22±0.10	0.04±0.00	0.04±0.00	-	.-	-	12.8±0.7	-	-	-	19.2±4.0^{b}	-	-	-
Day 7	0.28±0.15	0.05±0.01	0.04±0.01	0.26±0.07	0.04±0.01	0.05±0.01	12.2±1.5	13.2±1.2	13.3±1.0	13.2±0.6	21.3±11.7^{ab}	20.3±9.7^{ab}	25.1±8.1^{b}	24.6±4.2^{c}
Day 14	0.22±0.06	0.04±0.00	0.05±0.01	0.22±0.02	0.05±0.00	0.03±0.01	13.2±1.9	14.2±1.7	14.3±1.1	12.8±1.8	27.5±13.7^{ab}	25.5±10.7^{ab}	34.6±7.8^{ab}	24.4±9.1^{bc}
Day 28	0.23±0.02	0.08±0.01	0.06±0.02	0.23±0.00	0.06±0.01	0.06±0.02	12.5±1.1	13.5±0.9	14.0±1.2	14.1±1.1	19.5±4.8^{ab}	20.5±5.2^{b}	32.1±8.3^{ab}	34.0±11.1^{abc}
Day 35	0.19±0.04	0.07±0.00	0.08±0.01	0.19±0.02	0.07±0.00	0.07±0.01	13.1±1.6	12.1±1.4	14.2±1.3	15.3±0.7	28.3±11.4^{ab}	25.4±9.3^{ab}	35.7±11.0^{ab}	44.4±4.0^{a}
Day 42	0.20±0.05	0.04±0.01	0.06±0.02	0.19±0.02	0.04±0.01	0.06±0.02	14.3±2.4	14.5±2.2	14.3±1.3	14.7±1.5	39.0±15.3^{a}	32.9±10.3^{ab}	36.9±9.9^{ab}	39.6±12.3^{abc}
Day 56	0.20±0.03	0.05±0.01	0.08±0.00	0.20±0.01	0.05±0.01	0.09±0.00	13.7±0.7	13.0±0.5	14.9±1.1	15.0±1.2	33.0±4.1^{a}	34.2±5.1^{a}	39.8±6.3^{a}	42.0±10.5^{ab}

2.3. Total mercury and organic mercury

Total and organic mercury (T-Hg and MeHg) contents were analysed in each specimen muscle and liver tissues, as well as in seawater samples (to eliminate the possibility of Hg exposure via water; results were always below the detection limits; see also **Annex 2, Table A.2.2.**), whereas pooled samples were used for brain tissue (3 pools per sampling point, $n = 2$ per pool) due to the insufficient amount of sample to undertake analysis per specimen. Methylmercury contents were also determined in the feeds (control and MeHg-enriched) throughout the experiment revealing stable levels (results given in **Annex 2, Table A.2.2.**). Methylmercury was extracted from the samples (fish and feeds) as described by Scerbo and Barghigiani (1998), i.e. freeze-dried samples (approximately, 200 mg) were hydrolyzed in 10 ml of hydrobromic acid (47% w/w, Merck), followed by MeHg extraction with 35 mL toluene (99.8% w/w, Merck) and toluene removal with 6 ml cysteine aqueous solution (1% L-cysteinium chloride in 12.5% anhydrous sodium sulfate and 0.775% sodium acetate; Merck). Then, total Hg and MeHg were determined in samples (10-15 mg for solids or 100-200 µL for liquids) by atomic absorption spectrometry (AAS), following the method 7473 of the US EPA (2007), using an automatic Hg analyser (AMA 254, LECO, USA). Mercury concentrations were calculated from linear calibration (using, at least, five different standard concentrations), with a Hg(II) nitrate standard solution (1000 mg L^{-1}, Merck) dissolved in nitric acid (0.5 mol L^{-1}, Merck), and the detection limit was 0.005 mg kg^{-1}, wet weight (ww). Accuracy was checked through the analysis of the certified reference material DORM-4 (fish protein certified reference material for trace metals, National Research Council Canada, Canada), and results obtained in the present study were within the certified range of values (T-Hg: certified value = 0.410 ± 0.055 mg kg^{-1}, value obtained in the present work = 0.390 ± 0.025 mg kg^{-1}; MeHg: certified value = 0.354 ± 0.031 mg kg^{-1}, value obtained in the present work = 0.353 ± 0.062 mg kg^{-1}). A minimum of three measurements (replicates) were performed per sample, and results were reported as mg kg^{-1} ww, according to sample moisture content (results shown in **Annex 2, Table A.2.3.**). Blanks were always tested in the same conditions as the samples. Prior to utilization, all laboratory ware was cleaned with nitric acid (20% v/v) for 24h and rinsed with ultrapure water to avoid contamination. All standards and reagents were of analytical (*pro analysi*) or superior grade.

2.5. Data analysis

The percentage of MeHg lost during the elimination phase of the experiment, i.e. the elimination factor (EF) was calculated according to the equation: $EF(\%) = 100 - [(C_{end} / C_{initial}) \times 100]$, where C_{end} is the average concentration (mg kg^{-1}) in specimens from each treatment sampled at days 35, 42 and 56, and $C_{initial}$ is the average concentration (mg kg^{-1}) in specimens by the end of the exposure phase, i.e. day 28 (Jebali et al., 2014). The EF was considered to be 0 whenever

C_{end} was higher than $C_{initial}$. Differences between experimental treatments (biometric data, MeHg contents, %MeHg and EF) or tissues (MeHg contents and %MeHg) were examined using the analysis of variance one-way nested ANOVA. Data were transformed, whenever necessary, to comply with the assumptions of normality (Kolmogorov–Smirnov's test) and homogeneity of variances (Levene's test) required to perform this analysis. Subsequently, the post-hoc Tukey HSD test was carried out to spot significant differences. Additionally, two-way ANOVA was performed in order to detect significant differences in MeHg contents and %MeHg between sampling days and treatments. Statistical analyses were performed at a significance level of 0.05, using STATISTICA™ software (Version 7.0, StatSoft Inc., Tulsa, Oklahoma, USA).

3. Results and Discussion

3.1. Starting point and control treatments

In the beginning of the experiment (day 0), higher MeHg contents were observed in juvenile seabass muscle tissues compared to those found in brain and liver (muscle: 0.22 ± 0.10 mg kg^{-1}; brain and liver: 0.04 ± 0.00 mg kg^{-1}; statistical differences shown in **Table 2.1.2.**). Methylmercury was the predominant form of Hg in the muscle (i.e. 81.1 ± 0.1 %) whereas lower MeHg percentages in relation to T-Hg (%MeHg) were observed in the remaining tissues (liver: 61.7 ± 1.8 %; brain: 21.8 ± 0.1 %; statistical differences shown in **Table 2.1.2.**).

In the following days of the experiment (days 7-56) MeHg contents and %MeHg in muscle tissues of control specimens (18_control and 22_control) remained similar to those found in day 0, despite the significant increase in animal weight during the experiment (**Figure 2.1.2.**; **Table 2.1.1.**; $p < 0.05$). In contrast, a slight increase in MeHg content was found in brain and liver, as well as in %MeHg (maximum values, i.e. 36.4% and 65.8% at day 35, in brain and liver, respectively; **Figures 2.1.2.D,E,F**). Apart from intraspecific variability between specimens, a possible explanation for such MeHg increase in brain and liver tissues could be related to the small, but not negligible, amount of MeHg in the control diet (see also **section 2.1.**) due to the incorporation of fish meals and oils in the feed. Therefore, a low-level exposure to MeHg, even at trace concentrations existed during the acclimation, exposure and elimination phases. A similar trend was also observed in a study performed with neotropical fish *Hoplias malabaricus* exposed to dietary MeHg, i.e. a very low, but chronic exposure to MeHg before initiating the experiment, resulting in MeHg bioaccumulation in fish liver and kidneys from the non-contaminated group (Mela et al., 2007). Despite the different trends of each sampled tissue, throughout time, MeHg contents and %MeHg in fish exposed to low levels of MeHg contamination (equivalent to approximately 0.1 mg kg^{-1} ww) were not significantly different within temperature exposures in control treatments (i.e. 18_control and 22_control; **Figure 2.1.2.**).

3.2. Temperature exposure and MeHg bioaccumulation (MeHg-enriched treatments)

During the exposure phase (sampling days 7 to 28), MeHg contents were significantly affected by the sampling day and treatment (two-way ANOVA, F = 1.33, $p = 0.23$, $p < 0.001$; **Figure 2.1.2.; Table 2.1.3.**), revealing a significant interaction between these variables in all tissues. In fact, significant differences between control specimens (18_control and 22_control) and contaminated ones (i.e. 18_MeHg-enriched and 22_MeHg-enriched) were observed in MeHg contents immediately after day 7 ($p < 0.05$; **Figures 2.1.2.A,B,C**).

Fish exposed to a warmer temperature (i.e. 22_MeHg-enriched) exhibited, in general, higher MeHg contents in the three tissues than fish exposed to the lower temperature (18 ºC; 18_MeHg-enriched), even though the same amount of feed was ingested. These results seem to agree with the fact that environmental and body temperatures can affect the entry of chemical contaminants in the body, as well as contaminant detoxification mechanisms, thus promoting changes in contaminant bioaccumulation patterns (Gordon, 2003, Siscar et al., 2014). A recent field study performed with an estuarine fish (*Fundulus heteroclitus*) also revealed higher MeHg levels in fish naturally exposed to elevated temperatures (Dijkstra et al., 2013), likely as a consequence of an increase in organisms metabolism.

Table 2.1.2. Statistical comparisons (one-way ANOVA and post-hoc Tukey test) between muscle, liver and brain (MeHg contents and %MeHg) during the experiment. Asterisks indicate significant differences ($p < 0.05$; $df = 15$) Abbreviations: %MeHg - 18_control – seawater temperature set at 18 °C and animals fed with control diet; 18_MeHg-enriched – seawater temperature set at 18 °C and animals fed with MeHg-enriched diet; 22_control – seawater temperature set at 22 °C and animals fed with control diet; 22_MeHg-enriched – seawater temperature set at 22 °C and animals fed with MeHg-enriched diet.

		Day 0	**Day 7**	**Day 14**	**Day 28**	**Day 35**	**Day 42**	**Day 56**
MeHg content	One-way ANOVA	MS = 0.0032; F = 7.94; p = 0.0404*	MS = 0.0842; F = 1.04; p = 0.3654	MS = 0.5797; F = 2.89; p = 0.0817	MS = 8.68; F = 9.51; p = 0.015*	MS = 12.86; F = 8.38; p = 0.0024*	MS = 5.80; F = 10.42; p < 0.001*	MS = 10.00; F = 15.83; p = 0.01*
	Muscle x Liver	0.0337*	0.9785	0.8241	0.4158	0.986	0.1431	0.0015*
	Muscle x Brain	0.0329*	0.3986	0.1506	0.0062*	0.0111*	0.0512	0.5501
	Liver x Brain	0.9301	0.3237	0.3257	0.0559	0.0147*	0.0017*	0.0009*
%MeHg	One-way ANOVA	MS = 2020.8; F = 308.7; p < 0.001*	MS = 1162.5; F = 67.18; p < 0.001*	MS = 740.9; F = 20.51; p < 0.001*	MS = 102.1; F = 5.89; p = 0.0108*	MS = 1082.2; F = 52.65; p < 0.001*	MS = 928.9; F = 125.2; p < 0.001*	MS = 740.9; F = 20.51; p < 0.001*
	Muscle x Liver	0.0005*	0.0001*	0.0001*	0.0457*	0.0001*	0.0001*	0.0002*
	Muscle x Brain	0.0003*	0.0744	0.4778	0.8251	0.6935	0.0049*	0.3816
	Liver x Brain	0.0056*	0.0002*	0.0002*	0.0361*	0.0002*	0.0001*	0.0142*

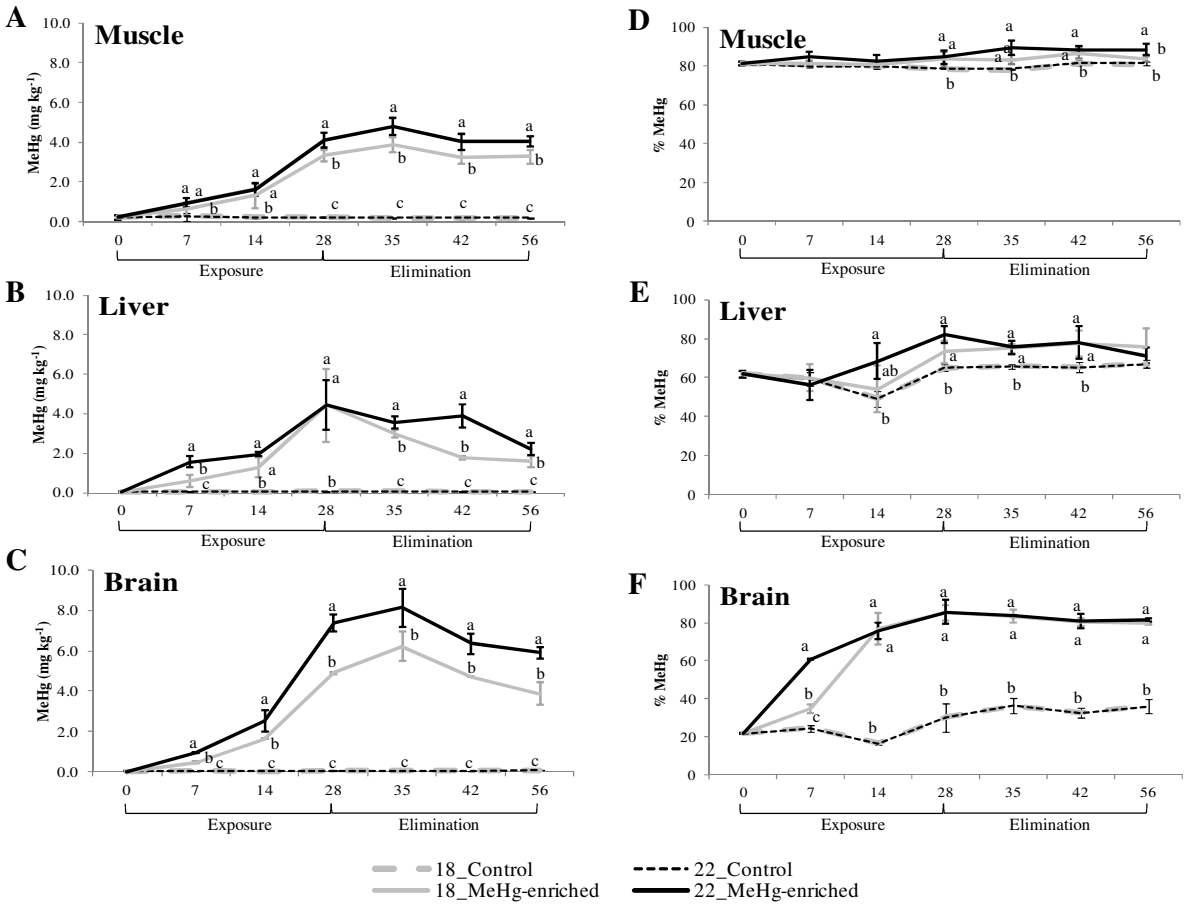

Figure 2.1.2. Methylmercury (MeHg) concentrations (mg kg^{-1} of wet weight) and %MeHg (%) in three tissues (muscle, liver and brain) of juvenile seabass (mean ± standard deviation; n = 6) sampled from each treatment, during the trials. Different letters indicate significant differences between sampling days ($p < 0.05$; $df = 15$). Abbreviations: 18_control – seawater temperature set at 18 °C and animals fed with control diet; 18_MeHg-enriched – seawater temperature set at 18 °C and animals fed with MeHg-enriched diet; 22_control – seawater temperature set at 22 °C and animals fed with control diet; 22_MeHg-enriched – seawater temperature set at 22 °C and animals fed with MeHg-enriched diet.

Despite the overall MeHg increase in fish from treatment 22_MeHg-enriched, different MeHg accumulation trends were observed between the three tissues (**Figure 2.1.2.**), most likely due to distinct metabolism and blood supplies of each fish organ (Becket et al., 2007; Siscar et al., 2014). For instance, in the muscle tissue (**Figure 2.1.2.A**), significant differences between 18_MeHg-enriched and 22_MeHg-enriched were only registered at day 28 ($p < 0.05$), whereas in liver and brain statistical significance between these treatments was obtained immediately at day 7 (**Figures 2.1.2.B,C**). Noteworthy and contrasting the other two tissues, in the liver tissue statistical differences between 18_MeHg-enriched and 22_MeHg-enriched were not observed at day 28 (**Figure 2.1.2B**).

Comparing MeHg levels in tissues of MeHg-enriched fish (**Figures 2.1.2.A,B,C**; **Table 2.1.2.**), brain registered the highest MeHg contents (4.89 ± 0.06 mg kg^{-1} ww and 7.36 ± 0.42 mg

kg^{-1} ww at day 28 in 18_MeHg-enriched and 22_MeHg-enriched, respectively), followed by liver (4.42 ± 1.83 mg kg^{-1} and 4.46 ± 1.25 mg kg^{-1}, respectively) and muscle (3.35 ± 0.29 mg kg^{-1} and 4.10 ± 0.36 mg kg^{-1}, respectively). Previous research on dietary MeHg exposure in fish has also reported higher accumulation in the brain compared to liver and muscle (e.g. Korbas et al., 2012; Amlund et al., 2015), likely because the blood–brain barrier is a tissue relatively permeable to MeHg, thus, emphasising MeHg potential for neurotoxicity (e.g. Korbas et al., 2012).

Table 2.1.3. Results of two-way ANOVA evaluating the effects of time (sampling days 7, 14, 28, 35, 42 and 56) and treatment (18_control – seawater temperature set at 18 °C and animals fed with control diet; 18_MeHg-enriched – seawater temperature set at 18 °C and animals fed with MeHg-enriched diet; 22_control – seawater temperature set at 22 °C and animals fed with control diet; 22_MeHg-enriched – seawater temperature set at 22 °C and animals fed with MeHg-enriched diet) on MeHg contents and percentages in relation to T-Hg contents (i.e. %MeHg) in seabass muscle, liver and brain. Asterisks indicate significant differences ($p < 0.05$; df = 15).

Parameters	MS	F	p
Muscle MeHg content			
Time	15.35	26.04	< 0.001*
Treatment	118.07	200.33	< 0.001*
Time x Treatment	5.64	9.57	< 0.001*
Muscle %MeHg			
Time	42.38	6.96	< 0.001*
Treatment	318.50	52.32	< 0.001*
Time x Treatment	16.04	2.64	0.003*
Liver MeHg content			
Time	9.94	8.54	< 0.001*
Treatment	84.1	72.25	< 0.001*
Time x Treatment	3.9	3.35	0.0015*
Liver %MeHg			
Time	516.38	12.88	< 0.001*
Treatment	396.11	9.88	< 0.001*
Time x Treatment	53.32	1.33	0.23
Brain MeHg contente			
Time	44.64	53.35	< 0.001*
Treatment	414.62	495.47	< 0.001*
Time x Treatment	9.3	11.11	< 0.001*
Brain %MeHg			
Time	689.72	44.83	< 0.001*
Treatment	8587.75	558.64	< 0.001*
Time x Treatment	143.26	9.31	< 0.001*

3.3. Temperature exposure and MeHg elimination (MeHg-enriched treatments)

In fish, MeHg is eliminated from the body mainly through the kidney, liver and possibly by the gills (Renfro et al., 1974). The process of MeHg elimination/detoxification was investigated

at two temperatures (18_MeHg-enriched and 22_MeHg-enriched; **Figures 2.1.2**. and **2.1.3.**; **Table 2.1.2.**). Different elimination patterns were observed in the three tissues. Overall, even though fish from contaminated treatments were fed with the control diet during the elimination phase, higher MeHg contents were registered at day 35 in the muscle and brain compared to those observed in the last exposure day, i.e. in day 28 (MeHg contents in 18_MeHg-enriched and 22_MeHg-enriched at day 35 were, respectively, in brain: 6.20 ± 0.72 mg kg^{-1} and 8.13 ± 0.94 mg kg^{-1}; muscle: 3.85 ± 0.88 mg kg^{-1} and 4.80 ± 0.45 mg kg^{-1}; **Figures 2.1.2.A,C**), which might be related to a redistribution of MeHg from the liver and kidneys to the other organs (Branco et al., 2011). Conversely, the liver presented a decrease in MeHg content straight after the first days of elimination (**Figure 2.1.2.B**), thus showing higher MeHg percentages of elimination (i.e. EF) during the clearance period (up to 50.3 ± 3.1% and 64.2 ± 4.5% in 18_MeHg-enriched and 22_MeHg-enriched, respectively; **Figure 2.1.3.B**), regardless of temperature exposure, most likely due to the fact that liver is a primary organ of contaminant redistribution to other tissues, elimination, detoxification and transformation (Yamashita et al., 2005; Wang et al., 2013). Yet, lower EFs were obtained in this tissue at the highest temperature exposure compared to the lowest one. Literature is still extremely scarce in what concerns the effect of temperature in metal elimination, limiting comparisons with previous findings, but a recent study suggested that warmer temperatures may favour the hepato-biliary route for metal excretion in teleosts, rather than the renal one (Siscar et al., 2014).

On the other hand, muscle revealed the lowest EFs (2.7 ± 0.2% and 2.0 ± 0.2% at day 56, in 18_MeHg-enriched and 22_MeHg-enriched, respectively; **Figure 2.1.3.A**), in agreement with the fact that this tissue usually exhibits extremely low MeHg elimination rates (e.g. Wang et al., 2013; Amlund et al., 2015). A recent study with zebra fish (*Danio rerio*) revealed a significant reduction of MeHg levels in the muscle only after 4 weeks of elimination and, even then, values were still above those of the control group of specimens (Amlund et al., 2015).

Finally, contrasting muscle and liver, brain of juvenile seabass subjected to a higher temperature revealed significantly higher EFs at day 42 (13.8% against 3.5% in 18_MeHg-enriched and 22_MeHg-enriched, respectively), but not at day 56 (near 20% in both temperature exposures; **Figure 2.1.3.C**). Such variation between days 42 and 56 could be due to the biphasic characteristic of Hg elimination from the brain, usually evidencing an initial rapid phase in which the decline in the body burden is associated with high levels of Hg being cleared from this tissue, followed by a slower phase of Hg clearance (Takahata et al., 1970).

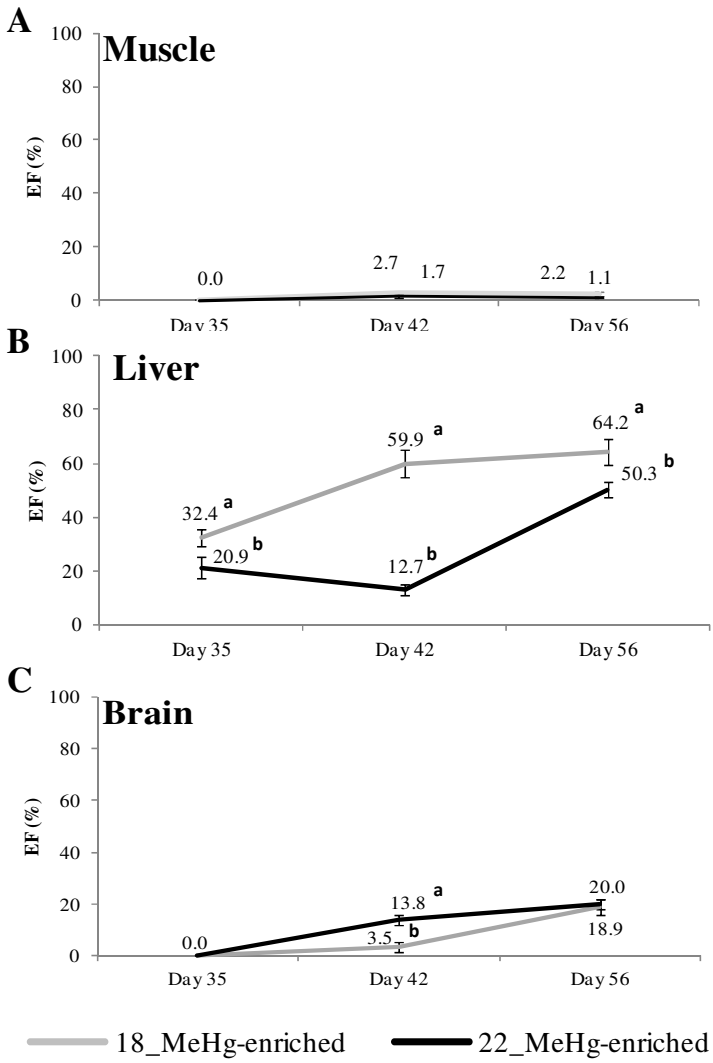

Figure 2.1.3. Percentages of eliminated MeHg (EF; %; n = 6) during the elimination phase (28 days) in contaminated specimens exposed to 18 ºC and 22 ºC, sampled from each treatment, during the trials. Different letters indicate significant differences between sampling days (p < 0.05; df = 12). Abbreviations: 18_MeHg-enriched – seawater temperature set at 18 °C and animals fed with MeHg-enriched diet; 22_MeHg-enriched – seawater temperature set at 22 °C and animals fed with MeHg-enriched diet.

3.4. Temperature exposure and relation between MeHg and T-Hg during the experiment (MeHg-enriched treatments)

Mercury speciation in biota, particularly in fish, is a process which still remains unclear and controversial. Under control seawater temperature conditions, some studies on Hg dietary exposure reported the conversion of inorganic Hg into MeHg (methylation) but not an evident demethylation of Hg (e.g. Wang et al., 2013), while others have suggested the occurrence of Hg demethylation at the liver and/or inter-organ transportation of Hg species (Collin et al., 2009; Huang et al., 2012; Wang et al., 2013). In what concerns the percentages of MeHg with respect

to T-Hg (%MeHg) in the present study, results were again very variable within the three tissues (**Figures 2.1.2.D,E,F**).

Even though fish were exposed to Hg in the MeHg form, during the exposure phase the increase in fish muscle MeHg content was proportionally accompanied by an increase of other forms of Hg (i.e. T-Hg), as the %MeHg in this tissue were only significantly higher than those in the beginning of the experiment at day 35 (only in treatment 22_MeHg-enriched, i.e. 89.2 ± 3.9%; **Figure 2.1.2D**). Furthermore, in this tissue significant differences between 18_MeHg-enriched and 22_MeHg-enriched were only observed at day 56 ($p < 0.05$). Following the increase in MeHg contents during the elimination phase (values at day 35 were higher than those at day 28; see **section 3.2**.), %MeHg have not significantly diminished despite fish were fed control diets in this period.

On the other hand, brain %MeHg significantly increased straight after the first days of exposure, reaching a value up to 4 times higher than the level found at the trial starting point, in both temperature exposures (18_MeHg-enriched: 85.2 ± 3.8%; 22_MeHg-enriched: 85.7 ± 6.5%; **Figure 2.1.2F**), suggesting that the brain is a potential primary target organ for MeHg bioaccumulation in fish (Pereira et al., 2014). Such potential can be related to the selectively permeable blood–brain barrier to contaminants in some fish species, hindering the transport of some (e.g. inorganic mercury) from the blood into the brain extracellular fluid, while being relatively permeable to others, such as MeHg (e.g. Korbas et al., 2012). Significant differences between %MeHg of 18_MeHg-enriched and 22_MeHg-enriched were only observed at day 7 (**Figure 2.1.2.F**). Contrasting the muscle, by the end of the elimination phase, the brain revealed a %MeHg decrease of about 4% and 2% in fish exposed to 18 ºC and 22 ºC, respectively, yet statistical significance between days 28 and 56 was only observed in treatment 18_MeHg-enriched ($p < 0.05$).

Finally, a different pattern was observed in the liver (**Figure 2.1.2.E**), i.e. %MeHg decreased in the first day of exposure and significantly increased only at day 28 in treatment 22_MeHg-enriched and at day 35 in treatment 18_MeHg-enriched. Such decrease could reflect the fact that a fraction of ingested MeHg was transformed into other Hg species and/or transported to organs such as muscle and brain until a threshold was reached in the liver, as previously discussed (Collin et al., 2009; Wang et al., 2013). By the end of the elimination phase, the higher discrepancy between MeHg and T-Hg contents in specimens from 22_MeHg-enriched promoted a significant decrease of %MeHg in this treatment (i.e. 82.1 ± 4.2% and 71.4 ± 4.0% in days 28 and 56, respectively), but not in 18_MeHg-enriched (i.e. %MeHg remained around 75%). Such differences could be related to temperature-induced alterations in the MeHg transformation process, as well as the following transportation of other forms of Hg from the liver to other organs (e.g. Huang et al., 2012.Wang et al., 2013). Hence, despite fish species are, to some extent, able to adapt to temperature variations, these results point out the importance of acquiring further

understanding of contaminants metabolization, speciation and kinetics considering environmental variations, such as those promoted by the expected climate change effects.

3.5. Linking climate change and potential impacts on seafood safety

Although little is known about the effect of global warming on the bioaccumulation of MeHg by marine species, the current findings indicate that higher seawater temperatures may increase MeHg bioaccumulation in biota's tissues and, on the other hand, it will also compromise their ability to eliminate contaminants. Such alterations in the way organisms cope with the presence of contaminants can lead to an increased human exposure to this contaminant through seafood consumption in a warming context, which will certainly raise concerns about consumers' safety and public health. Despite the fact that the presence of MeHg in seafood still remains unregulated (limits only set for T-Hg, i.e. 1 µg g^{-1} ww for predatory species and 0.5 µg g^{-1} ww for non-predatory species; EC, 2006), several reference values for MeHg exposure have been recommended by international bodies, such as the provisional tolerable weekly intake (PTWI) from FAO/WHO Expert Committee On Food Additives (FAO/WHO, 2003) of 1.6 µg of MeHg / kg body weight / week and the recent tolerable weekly intake (TWI) set by the European Food Safety Agency EFSA (2012) at 1.3 µg of MeHg / kg of body weight /week. Taking as example the present results, and selecting MeHg levels in seabass muscle from the worst-case result observed, i.e. the highest accumulation levels at 18 ºC and 22 ºC in day 35 (18_MeHg-enriched: 3.9 mg kg^{-1} ww and 22_MeHg-enriched: 4.8 mg kg^{-1} ww), the consumption of seafood exposed to a warmer seawater would increase human exposure to MeHg by about 20%. In other words, consumers would have a higher probability of exceeding the reference MeHg PTWIs or TWIs through seafood consumption in an ocean warming scenario, especially when consuming seafood species with MeHg concentrations close to regulated limits (T-Hg) and/or tolerable intakes (MeHg; e.g. top-predatory fish species). This perspective may be of particular concern in countries and/or populations where the *per capita* seafood consumption is high. Such expected increase of human health risks in a climate change context points out the need to allocate further research efforts in this area, addressing various chemical contaminants, as well as to revise and adapt the current recommendations/regulations for the presence of chemical contaminants in seafood.

4. Conclusions

This work evidenced how a variation of 4 ºC in seawater temperature may lead to significant changes in MeHg bioaccumulation and elimination processes in marine fish species, as well as its partitioning in different tissues like muscle, liver and brain tissues. Overall, temperature

increase triggered higher MeHg accumulation. In all treatments, the brain was the primary organ for MeHg bioaccumulation, emphasizing the neurotoxic characteristics of this contaminant, followed by liver and muscle. Considering EFs, liver's ability to eliminate this element was reduced in a warmer environment. In conclusion, the welfare and survival of marine organisms inhabiting contaminated areas may be highly affected under ocean warming, so further research on species ecophysiological responses to such stress synergisms (i.e. climate change and chemical contamination) should be undertaken. Preliminary insights on seafood safety in a warming environmental context were also obtained with this study, pointing out that, if the average seawater temperature keeps rising as forecasted, consumers might face higher risks of exposure to MeHg through seafood consumption. Thus, further research should target other chemical contaminants and the influence of climate change on consumer safety, so that consumption recommendations and legislation on the presence of contaminants in seafood are adapted to the ocean of tomorrow.

Ethical statement

Fish trials were approved by the Ethical Committee of the Faculty of Sciences of the Lisbon University and were conducted in accordance with legal regulations (EU Directive 2010/63).

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Part 2.

Ecophysiological responses of juvenile seabass (*Dicentrarchus labrax*) exposed to increased temperature and dietary methylmercury

Manuscript 2.

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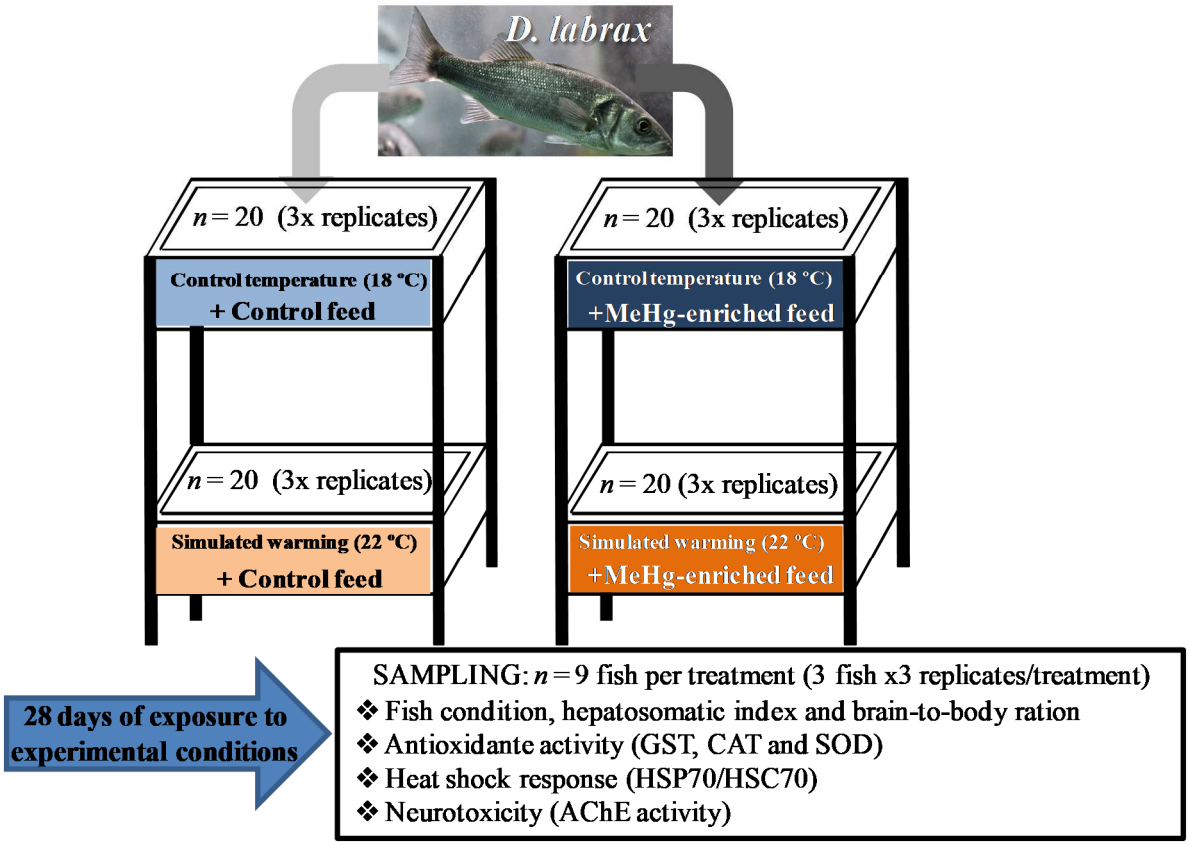
Graphical abstract



Abstract

The ecotoxicological effects of methylmercury (MeHg) exposure have been intensively described in literature. Yet, it is still unclear how marine biota will respond to the presence of MeHg under climate change, namely ocean warming. The present study aimed to investigate, for the first time, fish condition [Fulton's K index (K), hepatosomatic index (HSI) and brain-to-body mass ratio (BB_{ratio})] and several stress-related responses in an ecologically and commercially important fish species (*Dicentrachus labrax*) exposed for 28 days to dietary MeHg (8.0 mg kg^{-1} dw) and temperature increase (+4 ºC). Results showed significant impairments on fish condition, i.e. up to 34% decrease on K, >100% increase on HSI and 44% decrease on BB_{ratio}, compared to control conditions. Significant changes on tissue biochemical responses were observed in fish exposed to both stressors, acting alone or combined, evidencing the relevance of assessing possible interactions between different environmental stressors in ecotoxicological studies. For instance, muscle showed to be the least affected tissue, only revealing significant alterations in GST activity of MeHg-enriched fish. On the other hand, liver exhibited a significant induction of GST (>100%) and CAT (up to 74%) in MeHg-enriched fish, regardless of temperature exposure, as well as decreased SOD activity (19%) and increased HSP70/HSC70 content (87%) in fish exposed to warming alone. Brain showed to be affected by temperature (69% of GST inhibition and >100% of increased CAT activity), MeHg (>100% of increased CAT activity, 47% of SOD inhibition and 55% of AChE inhibition), as well as by the combination of both (GST, SOD and AChE inhibition, 17%, 48% and 53%, respectively). Hence, our data provides evidence that the toxicological aspects of MeHg can be potentiated by warmer temperatures, thus, evidencing the need for further research combining contaminants exposure and climate change effects, to better forecast ecological impacts in the ocean of tomorrow.

Keywords: dietary MeHg, seawater warming, animal condition, oxidative stress, heat shock, neurotoxicity.

1. Introduction

Anthropogenic derived impacts, such as the cumulative emissions of greenhouse gases and the introduction of pollutants in the environment, have resulted in remarkable contamination and unequivocally contributed to warming at a global scale (IPCC, 2014). Coastal environments are particularly vulnerable to climate change and anthropogenic pollution, once they are naturally and frequently exposed to a wide range of environmental stressors (e.g. tidal changes, salinity and temperature fluctuations), as well as subjected to constant inputs of industrial, agricultural and domestic chemical wastes (Boldt et al., 2014).

Over the last 30 years, an increase of the sea surface temperature (SST) has been globally observed, with particular emphasis in the Northern hemisphere, and the most up-to-date projections indicating an average SST increase of 3.7 ºC by the year 2100 (scenario RCP8.5, IPCC, 2014). Increased temperatures may lead to deleterious effects over marine organisms, for instance, promoting metabolic depression (Aurelio et al., 2013), as well as, notorious changes in tissues' heat shock response (e.g. HSP70/HSC70) and antioxidant machinery (e.g. CAT, SOD, GST), which play a key role in cell defence against the formation of reactive oxygen species (ROS) induced by stress (Madeira et al., 2013). Thus, the ability of marine species to acclimate or even adapt (or not) to rising temperatures will pose major challenges at a species, community and population level (Schiedek et al., 2007; Pörtner and Knust, 2007).

On the other hand, environmental warming may also result in higher availability and toxicity of marine pollutants, not only by facilitating their release from sediments and altering their chemistry, but also by affecting contaminants uptake/detoxification rates, as well as the metabolic rates and enzymatic activity of marine organisms (e.g. Schiedek et al., 2007; Noyes et al., 2009; Marques et al., 2010). Although literature is still limited, recent studies provide empirical proof to sustain that marine species' propensity to accumulate chemical contaminants might be exacerbated in a warmer environment (Dijkstra et al., 2013; Siscar et al., 2014; Maulvault et al., 2016). Such trend was evidenced in our recent work performed with *Dicentrarchus labrax* juveniles exposed to methylmercury (MeHg) through dietary sources, with specimens exposed to warmer temperatures exhibiting higher contents of MeHg in muscle, liver and brain, along with diminished ability to eliminate this element at the liver level (Maulvault et al., 2016). Despite being a contaminant of priority concern, up until now, little attention has been paid to the toxicological aspects and ecological implications of dietary MeHg exposure. Furthermore, the way marine vertebrate species will cope with its presence while dealing also with other environmental stressors, such as climate change effects, is still unclear. Therefore, it is of paramount importance to undertake research in this innovative and poorly understood field.

European seabass (*D. labrax*) is as top-predatory species, thus, being susceptible to accumulate high levels of biomagnifying pollutants, such MeHg (e.g. Miniero et al., 2013). Additionally, by

inhabiting coastal areas (wild specimens) or often being reared in offshore aquaculture systems (farmed specimens), which are particularly vulnerable to hydrographic alterations, it is expected that climate change effects may pose great ecological and toxicological challenges to this fish species (Marques et al., 2010; Rosa et al., 2012). These ecological features along with the great economical value of *D. labrax*, both wild or farmed (FEAP, 2013; FAO, 2014), make this species a suitable biological model to assess the possible impacts of climate change and chemical contamination in marine ecosystems.

In this context, our work aimed to assess the effects of increased temperature (+4 ºC) and MeHg dietary exposure (8.0 mg kg^{-1}), when acting alone or in combination, on the ecophysiological responses (fish condition, hepatosomatic index and brain-to-body mass ratio, antioxidant enzymes activities – GST, CAT and SOD, heat shock protein concentration and AChE activity) of juvenile seabass *D. labrax* (brain, muscle and liver).

2. Materials and methods

2.1. Control and MeHg-enriched diets

Non-contaminated (control) and MeHg-enriched diets with the same nutritional composition were manufactured by a specialized feed producing company (SPAROS Lda, Olhão, Portugal), considering the nutritional requirements of juvenile seabass (detailed feed composition can be consulted in **Annex 3, Table A.3.1.**; adapted from Maulvault et al., 2016). Feed preparation, as well as the methodologies used to determine its proximate chemical composition were performed as previously described in detail in Maulvault et al. (2016). For the MeHg-enriched diet, MeHg(II) chloride (CH_3ClHg, 99.8%, Sigma-Aldrich) was dissolved in a small volume of ethanol (< 6 mL, 100% v/v) and then mixed with the oils before pellets extrusion. Diets were maintained at 4 °C, and MeHg stability in the diets was evaluated throughout the experiment, by quantification of total and MeHg contents in the two feeds, using an automatic Hg analyser (AMA 254, LECO, USA), as described in detail in Maulvault et al. (2016). Methylmercury concentration in control and MeHg-enriched diets were, in dry weight (dw), 0.60 ± 0.01 mg kg^{-1} and 8.02 ± 0.14 mg kg^{-1} (mean ± standard deviation, n = 3; see also **Annex 3, Table A.3.2.**, adapted from Maulvault et al., 2016), respectively, with the control diet representing a low Hg level [~0.12 mg kg^{-1} wet weight (ww)] and the MeHg-enriched diet representing a high Hg level (~1.6 mg kg^{-1} ww), commonly found in species inhabiting contaminated coastal areas, susceptible to accumulate Hg, and that are natural preys of juvenile seabass (e.g. Cardoso et al., 2014).

2.2. Experimental design and biological sampling

D. labrax specimens with similar biometric characteristics were reared until juvenile stage (12.8 ± 0.7 cm total length; 19.2 ± 4.0 g total weight; **Table 2.2.1.**) at the aquaculture pilot station of the Portuguese Institute for the Sea and Atmosphere (EPPO-IPMA, Olhão, Portugal) using routine hatchery conditions. Subsequently, fish were transported to the aquatic facilities of "Laboratório Maritimo da Guia (MARE-FCUL, Cascais, Portugal)", where they were randomly and equitably distributed in 12 rectangular shaped incubating glass tanks (100 L each, total volume), within independent recirculating aquaculture systems. As previously described in Maulvault et al. (2016), each system was equipped with independent and automatic temperature (Frimar, Fernando Ribeiro Lda, Portugal) and pH control (model Profilux 3.1 N, GHL, Germany), protein skimmer (Reef SkimPro, TMC Iberia, Portugal), UV disinfection (Vecton 300, TMC Iberia, Portugal), biological filtration (model FSBF 1500, TMC Iberia, Portugal) and chemical filtration (activated carbon, Fernando Ribeiro Lda, Portugal). In order to avoid physiological stress related to high animal density, fish density was kept below 5 g body weight L^{-1}, within each incubation tank. On a daily basis, dead fish and faeces were removed and a 10% seawater renewal was performed in each incubation tank. Ammonia, nitrite and nitrate levels were daily checked, by means of colorimetric tests (Tropic Marin, USA), and kept below detectable levels, with the exception of nitrates, which were kept below 2.0 mg L^{-1}. Specimens were initially acclimated to laboratory conditions, during 30 days, being fed with the non-contaminated (control) diet (2% of average body weight, bw) and kept under the following abiotic conditions: i) dissolved oxygen (DO) > 5 mg L^{-1}; ii) temperature (TºC) = 18 ± 0.5 °C; iii) pH = 8.00 ± 0.10; iv) salinity = 35 ± 1 ‰ and v) photoperiod = 12L:12D (12 hours light:12 hours dark). Five days before initiating MeHg exposure, seawater temperature was slowly raised (1 ºC per day), until it reached 22 ºC in tanks simulating warming conditions (i.e. treatments 22_control and 22_MeHg-enriched, **section 2.3.**).

After the acclimation period, four treatments were carried out, each comprising three tanks or replicates (n = 20 animals per replicate/tank, i.e. 60 animals per treatment): i) 18_control (control conditions), i.e. seawater temperature set at 18 °C (i.e. average seawater temperature currently used in juvenile seabass rearing in the Iberian Peninsula) and animals fed with the control diet; ii) 18_MeHg-enriched, i.e. seawater temperature set at 18 °C and animals fed with the MeHg-enriched diet; iii) 22_control, i.e. seawater temperature set at 22 °C (i.e. simulating seawater warming, Δ = 4 °C according to IPCC projections scenario RCP8.5; IPCC, 2014) and animals fed with the control diet; iv) 22_MeHg-enriched, i.e. seawater temperature set at 22 ºC and animals fed with the MeHg-enriched diet. Specimens were 3x daily fed (2% bw), with the respective experimental feeds (control or MeHg-enriched), for a time period of 28 days. No mortality was

observed during the experimental trial. Seawater abiotic parameters were daily checked and adjusted to adequate levels whenever needed, as previously described.

After 28 days of dietary MeHg exposure, nine animals (three fish per tank/replicate) were randomly sampled from each of the 4 treatments (i.e. 36 animals collected in total). Fish trials were approved by the Ethical Committee of the Faculty of Sciences of the Lisbon University and conducted according to legal regulations (EU Directive 2010/63). Fish were euthanized by cervical sectioning, measured (±0.1 cm), weighted (±0.1 g), and dissected in order to remove brain, liver and muscle tissues. Fish tissues (approximately 200 mg of muscle and liver, and about 50 mg of brain) were sampled from each specimen, homogenized in ice-cold conditions with 1 mL of phosphate buffered saline (PBS; 140 mM NaCl, 3mM KCl, 10 mM KH_2PO_4, pH = 7.40 ± 0.02; reagents from Sigma-Aldrich, Germany), using an Ultra-Turrax® device (T25 digital, Ika, Germany). Afterwards, crude homogenates were centrifuged in 1.5 ml microtubes for 15 minutes at 10.000 x *g* and 4 ºC, supernatants were transferred to new microtubes, immediately frozen and kept at -80 ºC until further analyses. All analyses were, at least, performed in triplicate and using reagents of *pro analysis* grade or higher.

2.3. Biochemical analyses

2.3.1. Total protein content

Bradford assay (Bradford, 1976) was carried out in 96-well microplates (Nunc-Roskilde, Denmark) as to quantify total protein levels in each sample, and so that the subsequent biomarker results could then be normalized (i.e. given in mg of protein). Absorbance was read at 595 nm in a microplate reader (BioRad, Benchmark, USA). A calibration curve was generated using bovine serum albumin (BSA; Sigma Aldrich, Germany) at different dilutions (at least 7 different concentrations, ranging from 0 to 2 mg mL^{-1}) as standard.

2.3.2. Oxidative stress

Catalase (CAT)

Catalase activity (EC 1.11.1.6) was carried out following the procedure described by Johansson and Borg (1988), and adapted to 96-well microplates. A calibration curve was built using formaldehyde standards, with concentrations ranging from 5 to 75 µM of formaldehyde (Sigma Aldrich, Germany). Standard bovine catalase solution of 1523.6 $U \cdot mL^{-1}$ (Sigma Aldrich, Germany) was used as positive control. Enzyme activity was calculated considering that one unit of catalase is defined as the amount that will cause the formation of 1.0 nmol of formaldehyde per minute at 25 °C. Absorbance was read at 530 nm and results were presented as µM min^{-1} mg $protein^{-1}$.

Glutathione S-transferases (GST)

Glutathione S-transferases activity (EC 2.5.1.18) was determined essentially according to a method described by Habig et al. (1974) and adapted to 96-well microplates, using (1-Chloro-2,4-dinitrobenzene; 100 mM; Sigma-Aldrich, Germany) as substrate. Equine liver GST (Sigma-Aldrich, Germany) was used as standard and positive control. Absorbance was read at 340 nm every minute during 6 minutes, with the increase in absorbance being directly proportional to GST activity. Reaction rate was determined considering the molar CDNB extinction coefficient of $0.0053\varepsilon^{mM}$, and results were expressed as nmol min^{-1} mg $protein^{-1}$.

Superoxide dismutase (SOD)

Superoxide dismutase activity (EC 1.15.1.1) was carried out as described by Sun et al. (1988), using nitroblue tetrazolium (NBT) and xanthine oxidase (XOD) (both from Sigma-Aldrich, Germany). Superoxide dismutase from bovine erythrocytes (Sigma-Aldrich, Germany) was used as standard and positive control. Samples absorbance was read at 550 nm, and results were presented as the percentage of enzyme inhibition, using the following equation:

% inhibition = (Abs_{560}/min of negative control – Abs_{560}/min of sample) / (Abs_{560}/min of negative control) x 100

2.3.3. Heat shock response

Heat Shock Protein 70 (HSP70/HSC70) was quantified using an indirect Enzyme Linked Immunosorbent Assay (ELISA) based on a protocol from Njemini et al. (2005). The primary and secondary antibodies used in this assay were anti-Hsp70/Hsc70 (Acris, USA; diluted to 1.0 µg mL^{-1} in a 1% BSA solution) and anti-mouse IgG, fab specific, alkaline phosphatase conjugate (Sigma-Aldrich, Germany; also diluted to 1.0 µg mL^{-1} in 1% BSA), respectively. A calibration curve was performed using serial dilutions (at least 7 different concentrations) of purified HSP70 active protein (Acris, USA), ranging from 0 to 2 $\mu g \cdot mL^{-1}$ of protein. Absorbance was read at 405 nm and results were expressed in µg mg^{-1} protein.

2.3.4. Acetylcholinesterase (AChE) activity

Acetylcholinesterase (EC 3.1.1.7) activity was assessed in brain tissues, following the methodology first described by Ellman et al. (1961) and adapted to 96-well microplates. Samples absorbance was read at 412 nm, every minute during 10 minutes, and AChE activity was measured considering that one unit of enzyme is responsible for the formation of 1.0 µmol of thiocholine per minute. Results were expressed as nmol min^{-1} mg $protein^{-1}$.

2.4. Animal fitness

The Fulton's K index was directly calculated from the biometric data to determine fish condition, according to the formula:

$$K = 100W / TL^3$$

where W is the fish total weight (g) and TL is the total length (cm) (Ricker, 1975). The relationship between fish total weight and the respective organ weight was calculated for the liver (i.e. hepatosomatic index, HSI) and the brain (.i.e. brain-to-body mass ratio, BB_{ratio}), as to provide information on liver and brain condition, using the following equations:

$$HSI = \text{liver weight} / \text{total fish weight} \times 100$$

and,

$$BB_{ratio} = \text{brain weight} / \text{total fish weight} \times 100$$

2.5. Data analysis

Differences in biomarker levels were examined using the analysis of variance three-way ANOVA, using temperature exposure (18 ºC or 22 ºC), feed type (control or MeHg-enriched) and tissues (muscle, liver or brain) as variables. Significant differences in animal fitness indexes (K, HSI and BB_{ratio}), as well as, AChE levels (only assessed in the brain) were assessed using a two-way ANOVA instead (variables: temperature exposure and feed type). Data were Log-transformed, whenever necessary, to comply with the assumptions of normality (Kolmogorov–Smirnov's test) and homogeneity of variances (Levene's test) required for this analysis. Subsequently, the post-hoc Tukey HSD test was carried out to identify significant differences. Finally, potential correlations between biomarker levels and animal fitness indexes (K, HSI and BB_{ratio}) were performed by means of Pearson's correlation analysis. Statistical analyses were performed at a significance level of 0.05, using STATISTICA™ software (Version 7.0, StatSoft Inc., USA).

3. Results

Despite the generally higher size [i.e. total weight (W) and length (TL)] of fish exposed to warmer temperatures (i.e. 18_control: W = 19.5 ± 4.8 g and L = 12.5 ± 1.1 cm; 22_control: W = 32.1 ± 8.3 g and L = 14.0 ± 1.2 cm; 18_MeHg-enriched: W = 20.5 ± 5.2 g and L = 13.5 ± 0.9 cm; 22_MeHg-enriched: W = 34.0 ± 11.1 g and L = 14.1 ± 1.1 cm) after 28 days of MeHg exposure, no significant differences were observed in Fulton's K index (K) between the two experimental

temperatures, neither for non-contaminated and contaminated treatments ($p < 0.05$; **Figure 2.2.1.**). Yet, contaminated specimens (18_MeHg-enriched and 22_MeHg-enriched) revealed significantly lower K than non-contaminated fish (18_control and 22_control; **Figure 2.2.1.**; **Table 2.2.1.**). A similar trend was observed in BB_{ratio} but not in HSI, in which an increase of 85% and over 100% was observed in 22_MeHg-enriched and 18_MeHg-enriched, respectively, in relation to the reference conditions (i.e. 18_control; **Figure 2.2.1**; **Table 2.2.1**). A strong positive correlation was found between fish total weight and length ($r = 0.89$; $p < 0.05$; **Table 2.2.2.**), whereas K showed to be negatively correlated with HSI ($r = -0.68$; $p < 0.05$) but positively correlated with BB_{ratio} ($r = 0.76$; $p < 0.05$; **Table 2.2.2.**).

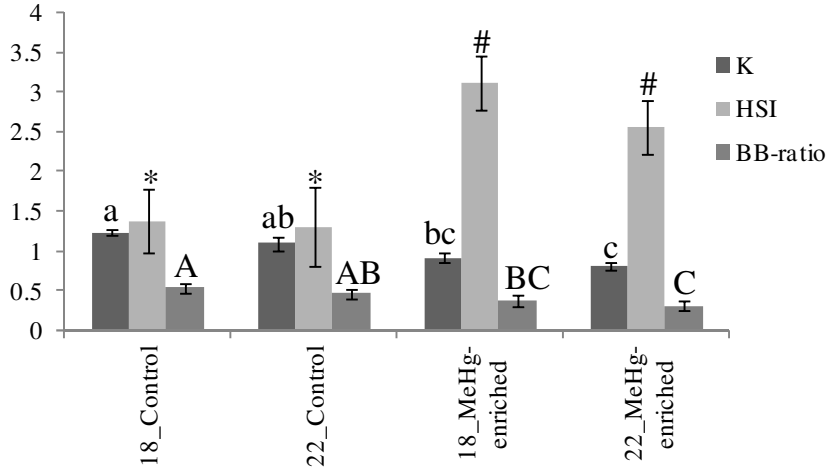


Figure 2.2.1. Fulton's condition index (K), hepatosomatic index (HSI) and brain-to-body mass ratio (BB_{ratio}) in *D. labrax* after 28 days of exposure (mean ± SD; $n = 9$). Different lowercase letters indicate significant differences in K between treatments ($p < 0.05$), whereas different uppercase letters indicate significant differences in BB_{ratio}, and different symbols (* and #) indicate significant differences in HSI. Abbreviations: 18_control – seawater temperature set at 18 °C and animals fed with the control diet; 18_MeHg-enriched – seawater temperature set at 18 °C and animals fed with the MeHg-enriched diet; 22_control – seawater temperature set at 22 °C and animals fed with the control diet; 22_MeHg-enriched – seawater temperature set at 22 °C and animals fed with the MeHg-enriched diet.

Table 2.2.1. Percentages of change in fish condition (K, HSI and BB_{ratio}), antioxidant enzymes (GST and CAT activity, and SOD inhibition), heat shock response (HSP70/HSC70) and brain AChE activity, in relation to values obtained in fish collected from control conditions (i.e. 18_Control). "↑" before the value indicates an increase compared to values found in treatment 18_Control, whereas "↓" indicates a decrease.

	Fish condition			Biomarkers					
	K	**HSI**	**BB-ratio**	**Tissue**	**GST**	**CAT**	**SOD**	**HSP70/HSC70**	**AChE**
	↓ 11%	↓ 5%	↓ 13%	Muscle	↑ >100%	↓ 11%	↑ 1%	↑ 37%	-
				Liver	↓ 41%	↓ 13%	↑ 19%	↑ 87%	-
				Brain	↓ 69%	↑ >100%	↑ 24%	↓ 6%	↑ 16%
ched	↓ 26%	↑ >100%	↓ 31%	Muscle	↑ >100%	↓ 23%	↑ 18%	↓ 1%	-
				Liver	↑ >100%	↑ 47%	↑ 29%	↓ 1%	-
				Brain	↑ 8%	↑ >100%	↑ 47%	↓ 10%	↓ 55%
ched	↓ 34%	↑ 85%	↓ 44%	Muscle	↑ 32%	↓ 24%	↑ 14%	↑ 26%	-
				Liver	↑ 96%	↑ 74%	↑ 23%	↑ 27%	-
				Brain	↓ 17%	↓ 1.5%	↑ 48%	↑ 9%	↓ 53%

Table 2.2.2. Pearson's correlation coefficients between biomarker levels and animal fitness indexes (K, HSI and BB_{ratio}). Asterisks indicate significant correlations between variables ($p < 0.05$). Abbreviations: Total length (TL), Total weight (W), Fluton's condition index (K), hepatosomatic index (HSI), brain-to-body mass ratio (BB_{ratio}).

	r
TL x TW	**0.89***
K x HSI	**-0.68***
K x BB_{ratio}	**0.76***
HSI x BB_{ratio}	**-0.88***
Muscle	
K x HSP	0.11
K x GST	-0.02
K x CAT	-0.48
K x SOD	-0.42
Liver	
K x HSP	-0.07
K x GST	0.20
K x CAT	**-0.67***
K x SOD	0.21
HSI x HSP	0.30
HSI x GST	-0.44
HSI x CAT	-0.53
HSI x SOD	-0.39
Brain	
K x HSP	0.18
K x GST	0.14
K x CAT	-0.50
K x SOD	**-0.61***
K x AchE	**0.72***
BB_{ratio} x HSP	-0.13
BB_{ratio} x GST	-0.18
BB_{ratio} x CAT	0.40
BB_{ratio} x SOD	**-0.63***
BB_{ratio} x AchE	**0.67***

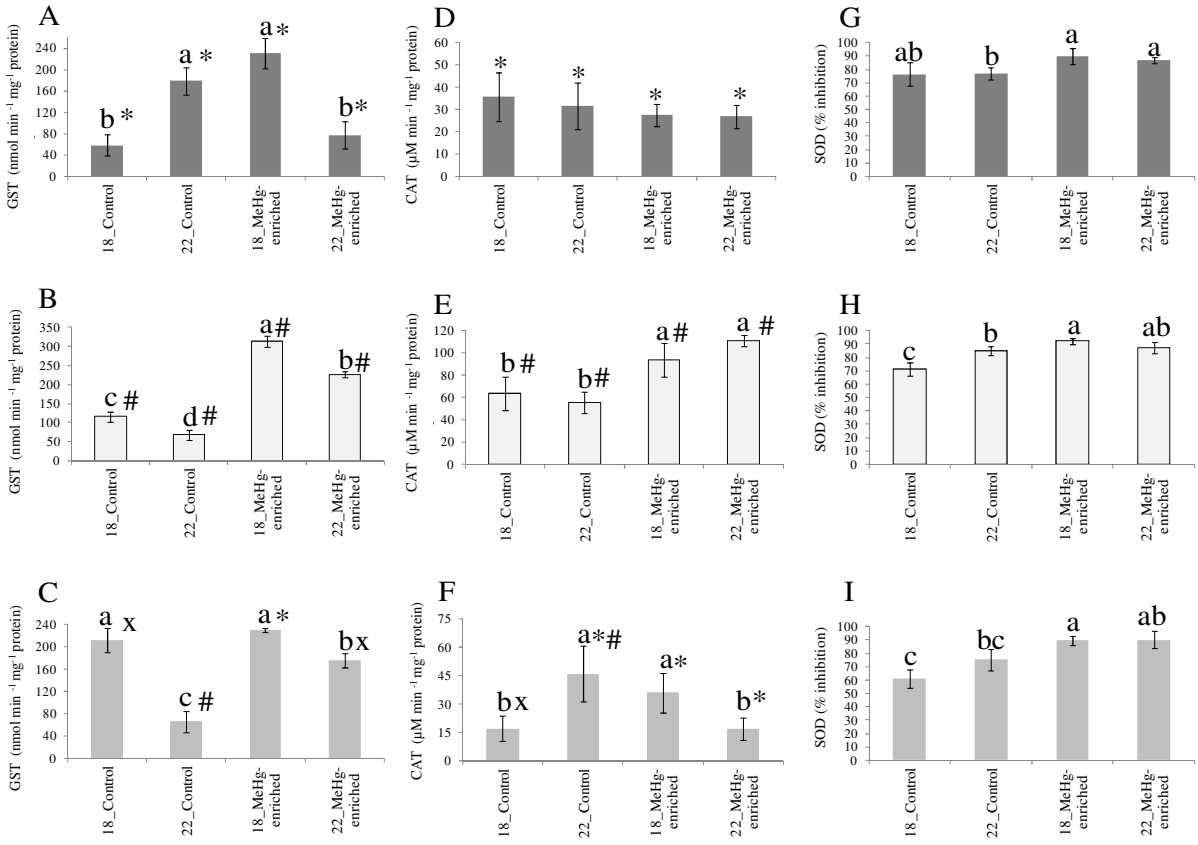


Figure 2.2.2. Glutathione S-transferases (GST; nmol min $^{-1}$ mg^{-1} protein) and catalase (CAT; µM min $^{-1}$ mg^{-1} protein) and superoxide dismutase activity (SOD; % inhibition) in muscle (A, D, G), liver (B, E, H) and brain (C, F, I) in *D. labrax* after 28 days of exposure (mean ± SD; $n = 9$). Different letters indicate significant differences between treatments ($p < 0.05$), whereas different symbols (*, # and x) indicate significant differences between tissues in the same treatment. Abbreviations: 18_control – seawater temperature set at 18 °C and animals fed with the control diet; 18_MeHg-enriched – seawater temperature set at 18 °C and animals fed with the MeHg-enriched diet; 22_control – seawater temperature set at 22 °C and animals fed with the control diet; 22_MeHg-enriched – seawater temperature set at 22 °C and animals fed with the MeHg-enriched diet.

Figure 2.2.2. presents the levels of oxidative stress-related enzymes GST, CAT and SOD. In muscle tissues, increased temperatures induced the activity of GST in non-contaminated specimens (over 100%, in relation to 18_control), but decreased in MeHg-enriched fish (i.e. 230.9 and 77.6 nmol min^{-1} mg $protein^{-1}$ in 18_MeHg-enriched and 22_MeHg-enriched, respectively; $p < 0.05$; **Figure 2.2.2.A**; **Table 2.2.1.**). Conversely, CAT activity did not significantly vary among treatments, whereas differences in SOD inhibition percentages were only significant between 22_control and 22_MeHg-enriched ($p < 0.05$; **Figures 2.2.2.B**,**C**). Regarding liver tissues, both GST and CAT as well as SOD activities were significantly affected by MeHg dietary exposure, regardless of temperature, evidencing an induction of GST and CAT, but the inhibition of SOD ($p < 0.05$; **Figures 2.2.2.B**,**E**,**H**; **Table 2.2.1.**). Furthermore, warmer temperatures also impaired the activity of GST (both control and MeHg-enriched treatments) and SOD (only control treatment) in this tissue ($p < 0.05$). GST and CAT brain activities were significantly affected by temperature (GST: 69% and 17% decrease, CAT: >100% increase and 1.5% decrease, in 22_control and 22_MeHg-enriched, respectively), regardless of fish diet (**Figure 2.2.2.C,F; Table 2.2.1.**). On the other hand, significant differences in SOD activity were spotted between non-contaminated and MeHg-enriched specimens exposed to 18 ºC ($p < 0.05$), but not in those exposed to 22 ºC (**Figure 2.2.2.I**). Both GST and CAT activities were significantly different within fish tissues, i.e.: GST, 18_control and 22_MeHg-enriched - muscle ≠ liver ≠ brain; 22_control – muscle ≠ liver and brain; 18_MeHg-enriched - liver ≠ muscle and brain; CAT, 18_control - muscle ≠ liver ≠ brain; 22_control - muscle ≠ liver; 18_MeHg-enriched and 22_MeHg-enriched - liver ≠ muscle and brain; $p < 0.05$; **Figures 2.2.2.A-F**). Moreover, significant correlations were found between i) CAT *versus* K at the liver level ($r = -0.67$; $p < 0.05$; **Table 2.2.2.**), and between ii) SOD *versus* BB_{ratio} ($r = -0.61$, $p < 0.05$) and SOD *versus* K in the brain ($r = -0.63$, $p < 0.05$; **Table 2.2.2.**).

Heat shock response (HSP70/HSC70) did not show significant differences within treatments (muscle and brain; **Figures 2.2.3A,C**). However, in the liver, higher temperatures increased HSP70/HSC70 levels in non-contaminated fish (i.e. 87% increase in 22_control; **Table 2.2.1.**), but not in contaminated ones (**Figure 2.2.3.B**). Among tissues, significant differences were only found between liver and brain, concerning treatment 22_control (**Figure 2.2.3.**).

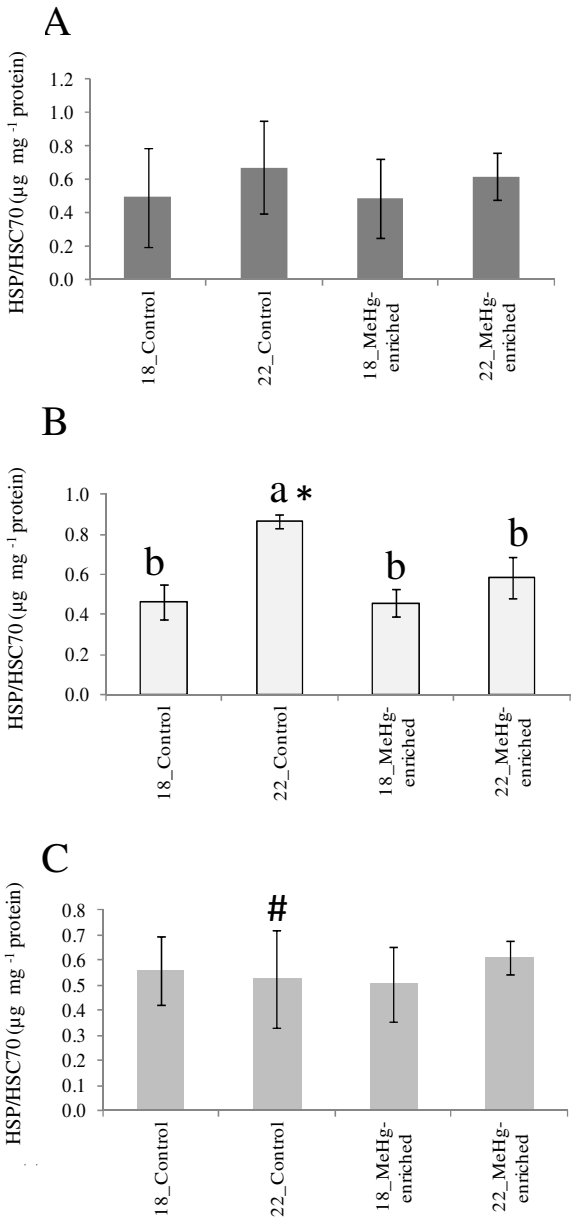


Figure 2.2.3. Heat shock protein HSP70/HSC70 concentration (µg mg^{-1} protein) in muscle (A), liver (B) and brain (C) in *D. labrax* after 28 days of exposure (mean ± SD; $n = 9$). Different letters indicate significant differences between treatments ($p < 0.05$), different symbols (* and #) indicate significant differences between tissues in the same treatment. Abbreviations: 18_control – seawater temperature set at 18 °C and animals fed with the control diet; 18_MeHg-enriched – seawater temperature set at 18 °C and animals fed with the MeHg-enriched diet; 22_control – seawater temperature set at 22 °C and animals fed with the control diet; 22_MeHg-enriched – seawater temperature set at 22 °C and animals fed with the MeHg-enriched diet.

Finally, AChE brain activity significantly decreased in MeHg dietary exposure treatments (over 50% at both temperature exposures; **Table 2.2.1.**), whereas rising temperature did not affect AChE activity (**Figure 2.2.4.**). Fish condition (K) and brain size (BB_{ratio}) were positively correlated with AChE ($r = 0.72$ and $r = 0.67$, respectively; $p < 0.05$; **Table 2.2.2.**).

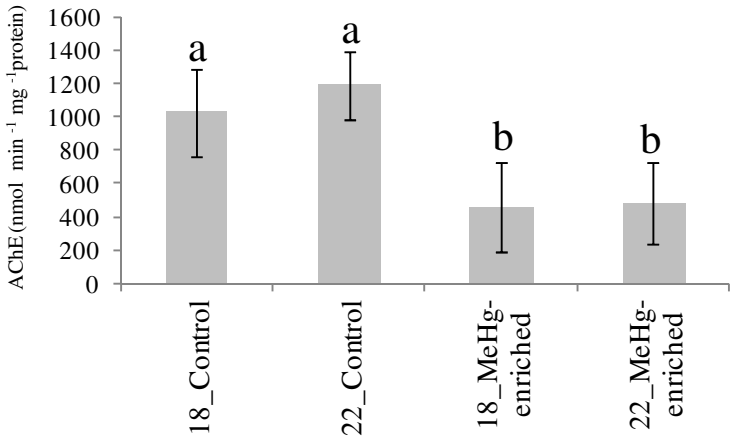


Figure 2.2.4. Acetylcholinesterase concentrations (AChE; nmol min^{-1} mg^{-1} protein) the brain tissues in *D. labrax* after 28 days of exposure (mean ± SD; $n = 9$). Different letters indicate significant differences between treatments ($p < 0.05$). Abbreviations: 18_control – seawater temperature set at 18 °C and animals fed with the control diet; 18_MeHg-enriched – seawater temperature set at 18 °C and animals fed with the MeHg-enriched diet; 22_control – seawater temperature set at 22 °C and animals fed with the control diet; 22_MeHg-enriched – seawater temperature set at 22 °C and animals fed with the MeHg-enriched diet.

4. Discussion

Studies combining different stressors are still very limited, both in field or laboratory conditions. Yet, the different trends observed in fish exposed to increased seawater temperature, dietary MeHg and the combination of both, highlighted the need to considerate the interactions between multiple stressors, and to fill this research gap in ecotoxicological studies. The interactions between temperature increase and MeHg exposure addressed in the current study showed that fish condition (K) can be negatively affected by MeHg exposure and further enhanced with a 4 ºC increase in seawater temperature, possibly due to changes in animal growth and metabolic rates (Dijkstra et al., 2013). Liver and brain are two primary organs for MeHg bioaccumulation (Maulvault et al., 2016), since the first is responsible for redistribution of contaminants to other tissues, as well as its elimination, detoxification and transformation (Yamashita et al., 2005; Wang et al., 2013), and the second is relatively permeable to MeHg (Korbas et al., 2012). Although a variety of factors (including physiological variations) may contribute to changes in liver size, HSI has been previously used as a toxicological biomarker, evidencing an increase in organisms exposed to contaminants, due to a higher activity of enzymes

which are responsible for contaminant biotransformation (Chen et al., 2001; Facey et al., 2005; Diniz et al., 2009). Such is the case of GST, which plays a key role in contaminant transformation, since it is a major second phase detoxification enzyme (e.g. Sheehan et al., 2001). Thus, the remarkably enhanced activity of this enzyme in liver tissues in MeHg-enriched specimens can be likely related to the intense contaminant biotransformation occurring in this organ. Increased GST activity following contaminant exposure has been previously reported in different fish species, namely in liver of *Oncorhynchus mykiss* (exposed to polychlorinated biphenyls commercial mixture; Pérez-López et al., 2002) and *Oncorhynchus kisutch* (exposed to Cd; Espinoza et al., 2012). As for the temperature effect, the inhibition of GST activity in fish exposed to +4 ºC (except in muscle from control specimens i.e. 22_control) seems to be in contradiction with the fact that antioxidant enzymes and their degradation products are extremely sensitive to temperature variations due to the formation of ROS, which generally leads to an increased activity of these enzymes. Yet, the mechanisms underlying the action of antioxidant enzymes are strongly species-dependent, mostly due to the distinct thermal tolerance limits of each species, as recently pointed in a study performed with five different estuarine species (Madeira et al., 2013).

Superoxide dismutase along with CAT, play a major defensive role against ROS that are induced by environmental stressors, the first converting superoxide radicals into hydrogen peroxide and molecular oxygen (O_2), while the second converts hydrogen peroxide into oxygen and water (Halliwell and Gutteridge, 1985). Previous field studies conducted in temperate marine organisms have evidenced how temperature can strongly mediate fish antioxidant machinery (e.g. Malanga et al. 2007; Madeira et al., 2016), and linked increased CAT and SOD with warmer seawater temperature, matching the present results in *D. labrax* brain CAT activity. Yet, for the same tissue, such trend was not observed when the warmer temperature was combined with MeHg exposure, likely due to the inability of the antioxidant enzyme machinery to compensate for the presence of both stressors when acting simultaneously. In fact, as previously reported, when excessive substrate is produced (negative feedback) or oxidative damage occurs, enzyme activity can be impaired (Carvalho et al., 2012; Karadag and Firat, 2014). The same principle could also justify SOD inhibition in fish exposed increased temperatures (liver), MeHg exposure (liver and brain) and the combination of both (liver and brain). In addition, SOD activity can also be impaired in the presence of metals, such as MeHg, since they can also directly bind to this enzyme, thus, resulting in diminished protective action against ROS formation and, ultimately, lipid peroxidation (Liebler and Reed, 1997; Carvalho et al., 2012). Noteworthy, the fact that CAT activity in the muscle and liver was not significantly increased by warmer temperatures, but rather by MeHg exposure (in liver), suggests that a 4 ºC increase in temperature (i.e. from the optimal 18 ºC to 22 ºC) may still fall into the thermal tolerance limits of juvenile *D. labrax*, thus, not being

sufficient to trigger CAT in both tissues, as it was also observed by, Madeira et al. (2013) in *D. labrax* muscle (i.e. CAT only significantly inducted at temperatures above 30 ºC).

In what concerns HSPs, several studies performed with fish species have reported an induction of these proteins when temperature fluctuations occur (e.g. Madeira et al. 2013, 2015), as well as in the presence of chemical contaminants (e.g. Sanders, 1993; Vijayan et al., 1998). Nonetheless, it has been recently evidenced that their use as ecotoxicological biomarkers can provide rather inconsistent and controversial data, because: i) various biotic and abiotic factors (e.g. gender, life stage, season, seawater, pH and salinity), as well as, synergisms with contaminants, may influence HSPs expression (Mahmood et al., 2014); ii) some species and/or tissues with high baseline HSP contents, may not reflect a significant induction of these proteins upon thermal stress (e.g. Botton et al. 2006; Madeira et al. 2015). Thus, the present study also pointed out to the inconsistency of this biomarker, as warmer temperatures significantly induced HSP70/HSC70 synthesis in the liver of non-contaminated fish but not in those exposed to MeHg, nor in the muscle and brain tissues.

Methylmercury neurotoxicological potential has been intensively described in the literature (e.g. Castoldi et al., 2001; Korbas et al., 2012; Amlund et al., 2015). As observed in the present study, one of the most notorious consequences of MeHg exposure in vertebrates is the reduction of the brain size, likely due to the fact that this neurotoxic contaminant can induce a significant decrease in the number of brain cells (Puga et al., 2016; Morccilo et al., 2016). Another effect is the inhibition of AChE activity (Jesus et al., 2013; Sampaio et al., 2016), due to the fact that this contaminant strongly binds to the AChE receptor (acetylcholine), thus blocking the electric signalling between neurons and the target cells. Such argument also supports the results presently observed in MeHg-enriched fish, regardless of temperature exposure. Changes in brain size, AChE activity and overall neurophysiological functioning have been associated with deleterious behavioural alterations in vertebrates, for instance, in terms of habitat selection, anxiety, judgment ability and locomotion, with dramatic ecological implications (Baatrup, 1991; Beauvais et al. 2001; Sampaio et al., 2016). In what concerns the effect of temperature exposure, Sampaio et al. (2016) reported a significant increase of AChE activity with warming in *Solea senegalensis* juvenile specimens, possibly due to increased fish metabolic rates. Yet, contrasting these authors, in the present study, increased temperature did not seem to enhance MeHg neurotoxicity.

5. Conclusions

Despite the dissimilar abilities of each tissue to cope with environmental stressors, the present work evidenced that fish antioxidant machinery (GST, CAT and SOD) is altered by increased temperature and MeHg, acting alone or in combination. Warmer temperature, by itself, was also responsible for triggering HSP70/HSC70 synthesis in the liver, while MeHg exposure (alone or combined with +4ºC of temperature increase) did not show a clear effect in fish heat shock

response. On the other hand, MeHg neurotoxic potential did not seem to be enhanced by warmer temperature. After 28 days of exposure to the experimental conditions, the notorious alterations of liver and brain morphometrics, as well as the changes occurred in fish biochemical responses suggest that, in the long term, species welfare and survival can be affected by both studied stressors, particularly for those in early and more vulnerable life stages (including larvae and juveniles).

To sum up, the present work provided preliminary insights on the possible impacts of climate change effects (rising seawater temperatures), particularly in coastal areas subjected to strong anthropogenic pressure and high levels of chemical contaminants, such as MeHg. Furthermore, the different patterns observed when fish are exposed to each stressor alone or in combination clearly emphasize the relevancy of assessing possible interactions between multiple stressors, especially when forecasting the impacts of climate change. As final take-home message, it is also worth noting the relevance of using a multi-biomarker approach in ecotoxicological studies, especially when considering the effect of multiple stressors and the interactions among them.

Ethical statement

Fish trials were approved by the Ethical Committee of the Faculty of Sciences of the Lisbon University and were conducted in accordance with legal regulations (EU Directive 2010/63).

Acknowledgments

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CHAPTER 3.

ANTI-INFLAMMATORY DRUGS: DICLOFENAC AS A CASE STUDY

Part 1.

Integrated multi-biomarker responses of juvenile seabass to diclofenac, warming and acidification co-exposure

Manuscript 3.

Maulvault, A.L., Barbosa V., Alves, R., Anacleto P., Camacho C., Cunha, S., Fernandes, J.O., Pousão Ferreira, P., Rosa, R., Marques, A., Diniz, M., 2018. Integrated multi-biomarker responses of juvenile seabass to diclofenac, warming and acidification co-exposure. *Aquatic Toxicology*, 202, 65–79. DOI: 10.1016/j.aquatox.2018.06.016.

Abstract

Pharmaceutical drugs, such as diclofenac (DCF) are frequently detected in the marine environment, and recent evidence have pointed out their toxicity to non-target marine biota. Concomitantly, altered environmental conditions associated to climate change (e.g. warming and acidification) can also affect marine organisms' physiology. Yet, the underlying interactions between these environmental stressors (pharmaceuticals exposure and climate change-related stressors) still require a deeper understanding. Comprehending the influence of abiotic variables on chemical contaminants' toxicological attributes provides a broader view of the ecological consequences of climate change. Hence, the aim of this study was to assess the ecotoxicological responses of juvenile seabass *Dicenthrachus labrax* under the co-exposure to DCF (from dietary sources, 500 ± 36 ng kg^{-1} dw), warming ($\Delta T^{o}C$ = +5 ºC) and acidification (ΔpCO_2 ~1000 µatm, equivalent to ΔpH = -0.4 units), using an "Integrated Biomarker Response" (IBR) approach. During 28 days, fish were exposed to these three stressors, acting alone or combined, in a full cross-factorial design, and blood, brain, liver and muscle tissues were subsequently collected in order to evaluate: i) animal/organ fitness; ii) haematological parameters and iii) molecular biomarkers. Results not only confirmed the toxicological attributes of DCF dietary exposure to marine fish species at the tissue (e.g. lower HSI), cellular (e.g. increased ENAs and lower erythrocytes viability) and molecular levels (e.g. increased oxidative stress, protein degradation, AChE activity and VTG synthesis), but also showed that such attributes are altered by warming and acidification. Hence, while acidification and/or warming enhanced some effects of DCF exposure (e.g. by further lowering erythrocytes viability, and increasing GST brain activity and Ub synthesis in muscle), the co-exposure to these abiotic stressors also resulted in a reversion/inhibition of some molecular responses (e.g. lower CAT and SOD inhibition and VTG synthesis). IBRs evidenced that an overall higher degree of stress (i.e. high IBR index) was associated to DCF and warming co-exposure, while the effects of acidification were less evident. The distinct responses observed when DCF acted alone or co-exposed with warming and acidification not only highlighted the relevance of considering the interactions between multiple environmental stressors in ecotoxicological studies, but also suggested that the toxicity of pharmaceuticals can be aggravated by climate change-related stressors (particularly, warming), thus, posing additional biological challenges to marine fish populations.

Keywords*:* Diclofenac, warming, acidification, multi-biomarkers, IBR, fish.

1. Introduction

Over the last decades, the consumption of pharmaceutical active compounds (PhACs) has increased drastically, reaching an average worldwide consumption of over 100,000 tons per year, and an average *per capita* consumption of 150 g per year in developed countries (Lonappan et al., 2016). As the removal of PhACs by waste water treatment plants (WWTPs) is still limited, many compounds and their metabolites are continuously introduced in the aquatic environment (concentrations ranging from ng L^{-1} up to low mg L^{-1}), potentially representing a risk to non-target marine species (e.g. Gros et al., 2012; Gaw et al., 2014; Mezzelani et al., 2017).

Widely known as one of the most popular "pain-killers", diclofenac (DCF; usually available in the forms of sodium or potassium salts) is a non-steroidal anti-inflammatory drug (NSAID) commonly prescribed to reduce inflammation and/or to relieve pain induced by different chronic diseases (e.g. arthritis) or injuries. Despite its intensive usage, in both humans (being in the top 50 list of most prescribed and sold pharmaceuticals; European Medicines Agency, 2013; ARSLVT, 2015) and stockbreeding (i.e. bovine and pork farming; European Medicines Agency, 2014), along with its inefficient removal at WWTPs (i.e. elimination of its parental form between 30 and 70%, and levels in wastewater samples in the order of µg L^{-1}; Lonappan et al., 2016), DCF's presence in aquatic systems remains unregulated in the European Union. Still, the European Commission has recently placed DCF under the "Watch List" of emerging non-regulated aquatic pollutants, for which further monitoring and toxicological data is needed, to accurately estimate their ecological risks and decide, in the future, whether their presence in the environment (and seafood) should be regulated or not (proposed maximum allowable DCF concentration of 0.1 µg L^{-1} and 0.01 µg L^{-1} in freshwater and seawater respectively; EC Decision 2015/495).

Although several studies have recently pointed out DCF's toxicity to marine species (Gonzalez-Rey and Bebianno, 2014; Mezzelani et al., 2016; Gröner et al., 2017), the ecotoxicological implications of this compound are still far from being completely unveiled, particularly for two reasons. First, most studies carried out so far were focused on DCF exposure via water (e.g. Munari et al., 2016; Boisseaux et al., 2017; Gröner et al., 2017), disregarding other contaminant exposure pathways, such as dietary exposure (i.e. trophic transfer along the food chain), which can be particularly important in predatory fish species (Zenker et al., 2014). To the best of our knowledge, so far, only two studies have assessed the ecotoxicological implications of DCF dietary exposure (Guiloski et al., 2015; Ribas et al., 2016). Second, chemical pollution is not the sole environmental stressor that marine species are subject to, and information on the potential effects of other environmental stressors (e.g. seawater warming and acidification) that can particularly affect the bioavailability and toxicity of emerging contaminants is limited (Marques et al., 2010; Amiard-Triquet et al., 2015). So far, only two studies have accounted for

the interactive effects of abiotic stressors (both following DCF water exposure), one using the marine bivalve *Ruditapes philippinarum* under acidification (Munari et al., 2016), and the other using a freshwater fish species *Gasterosteus aculeatus* (adult individuals) under hypoxia (Lubiana et al., 2016). Yet, climate change effects can already be felt in some regions of the world and are expected to worsen in the coming 50-100 years, increasing seawater temperature as high as +5 ºC (i.e. ocean warming), as well as, increasing CO_2 partial pressure (pCO_2) up to 1000 µatm, thus, leading to a seawater pH drop (i.e. ocean acidification; IPCC 2014; McNeil and Sasse, 2016). Thus, gathering data of environmental pollutants in multi-stressors context is urgently needed, as it will provide a better estimation of the potential ecotoxicological implications of pollutants in tomorrow's ocean.

In the field of ecotoxicology, as well as, of climate change effects, the marine fish species *Dicenthrachus labrax* has been frequently used as a suitable model species (e.g. Hernández-Moreno et al., 2011; Maulvault et al., 2016, 2017; Barboza et al., 2018), given its ecological characteristics and economical value, i.e.: i) it is a predatory fish species, inhabiting temperate estuaries and coastal areas and, likely accumulating high levels of chemical contaminants (FAO, 2018) and, therefore, being a suitable bioindicator in studies following dietary exposure to chemical contaminants; ii) since *D. labrax* is a commercially valuable species, the deleterious effects of environmental stressors, particularly in its early life stages (including larvae and juveniles) can potentially affect species recruitment and overall ecological success, thus, certainly having negative impacts in the fisheries and aquaculture sectors.

Hence, given the current lack of empirical data on effects of PhACs dietary exposure and the potential interactions of these contaminants with climate change-related stressors, this study aimed to assess, for the first time, different ecotoxicological responses (i.e. animal condition, haematological parameters, genotoxicity, oxidative stress, heat shock response, protein degradation, endocrine disruption and neurotoxicity) induced by DCF dietary exposure (500 ng kg^{-1} dw), seawater warming (ΔTºC = +5 ºC) and acidification (ΔpCO_2 ~1000 µatm, equivalent to ΔpH = -0.4 units) in different tissues (brain, liver and muscle) and blood of the European seabass *D. labrax*. Since reaching a general conclusion regarding the severity of stressors can be a challenging process, especially when multiple endpoints and stressors' interactive effects are at stake, an "Integrated Biomarker Response" approach (IBR; Guerlet et al. 2010) was used in the present study, as their application constitutes a practical and robust tool used in field and laboratory studies (e.g. Serafim et al., 2012; Ferreira et al., 2015; Madeira et al., 2016a). Such approach allowed to combine the different biomarker responses observed at the different organization levels (animal, tissue and cell), thus, providing a novel, wider and integrative understanding of the ecological impact of DCF in tomorrow's ocean.

2. Materials and Methods

2.1. Control and DCF-contaminated diets

Non-contaminated feed (control, CTR feed) and DCF contaminated feed (DCF-enriched feed) with the same nutritional composition were manufactured by SPAROS Lda (Olhão, Portugal). Detailed feed composition can be consulted in **Annex 4, Table A.4.1**. Briefly, a control diet (CTR feed) was formulated to mimic a commercial fishmeal-rich formulation for juvenile marine seabass with 62% crude protein and 15% crude fat. All powder ingredients were grinded (<200 micron) using a micropulverizer hammer mill (Hosokawa Micron, SH1, The Netherlands). Ingredients and fish oil were then mixed accordingly to the target formulation in a paddle mixer (Mainca RM90, Spain), and the feed mixture was further humidified with 25% deionized water at room temperature. The diet was extruded at 1.0 mm by means of a low-shear extruder (P55, Italplast, Italy). Upon extrusion, the feed pellets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). A 10 kg batch of CTR feed was subsequently contaminated with DCF (DCF-enriched feed). To do so, diclofenac sodium salt ($C_{14}H_{10}C_{l2}NNaO_2$, >98% purity, Sigma-Aldrich) solubilized in ethanol was further mixed in fish oil (total volume of 150 mL), and this solution was top-coated to the pellets with a pressurized spraying container (standard flat-fan nozzle; size 250 micron; pressure 2.1 bar). Given the current lack of toxicological data on pharmaceuticals dietary exposure, a DCF nominal concentration of approximately 500 ng kg^{-1} on a dry weight basis (dw) was selected to assure that ecotoxicological responses were elicited during the timeline of the trials, corresponding to a value within the range of lowest observed effect concentration for dietary exposure (Guiloski et al., 2015; Ribas et al., 2016) and matching a value ~10 times higher than the DCF concentration commonly found in marine biota inhabiting contaminated coastal areas (e.g. Huerta et al., 2013; Vandermeersch et al., 2015). DCF was subsequently quantified in both control and DCF-enriched diets, following the previously optimized and validated method of Cunha et al. (2017). DCF final concentration in the DCF-enriched diet was 500 ± 36 ng kg^{-1}, whereas DCF was not detected in the control diet (detection limit in feed = 0.1 ng kg^{-1}; quantification limit in feed = 0.3 ng kg^{-1}).

2.2. Organisms and acclimation

D. labrax specimens (n = 168) with similar biometric characteristics were reared until juvenile stage (total length: 6.4 ± 0.4 cm; total weight 4.4 ± 0.2 g) in the aquaculture pilot station of the Portuguese Institute for the Sea and Atmosphere (EPPO-IPMA, Olhão, Portugal) using routine hatchery conditions. Subsequently, fish were transported to the facilities of "Laboratório Marítimo da Guia (MARE-FCUL, Cascais, Portugal)", where they were randomly and equitably distributed in 24 rectangular shaped incubating glass tanks (50 L each, total volume) within

independent recirculation aquaculture systems (RAS; i.e. each group of three tanks corresponded to one treatment, and 3 replicate tanks x 8 treatments = 24 tanks in total; **Figure 3.1.1.**; see the description of each treatment in **section 2.3.**). Each tank was equipped with protein skimmer (Reef SkimPro, TMC Iberia, Portugal), UV disinfection (Vecton 300, TMC Iberia, Portugal), biological filtration (model FSBF 1500, TMC Iberia, Portugal) and chemical filtration (activated carbon, Fernando Ribeiro Lda, Portugal) to maintain seawater quality. Dead fish and faeces were daily removed as well as 25% seawater renewal in each incubation tank. Ammonia, nitrite and nitrate levels were daily checked, by means of colorimetric tests (Tropic Marin, USA), and kept below detectable levels, with the exception of nitrates, which were kept below 2.0 mg L^{-1}. Fish density was kept below 5 g body weight L^{-1} in order to avoid physiological stress related to high animal density. Specimens were initially acclimated to laboratory conditions, during 30 days, being fed with CTR feed (2% average body weight, bw) and kept under abiotic conditions as close as possible to those of their natural habitat: i) dissolved oxygen (DO) > 5 mg L^{-1}; ii) temperature (T ºC) = 19.0 ± 0.5 °C; iii) pH = 8.00 ± 0.10 units; iv) salinity = 35 ± 1 ‰; and v) photoperiod = 14L:10D (14 hours light:10 hours dark). Temperature, pH, salinity and DO were daily checked using a multi-parameter measuring instrument (Multi 3420 SET G, WTW, Germany). Temperature and pH were adjusted whenever needed by means of: i) temperature - an automatic seawater refrigeration system (± 0.1 °C; Frimar, Fernando Ribeiro Lda, Portugal), as well as submerged digital thermostats (200W, V2Therm, TMC Iberia, Portugal); ii) pH - individual pH probes (GHL, Germany) connected to a computerized pH control system (± 0.1 pH units; Profilux 3.1N, GHL, Germany), which monitored seawater pH in each tank every 2 s, and adjusted whenever need, via submerged air stones, by injecting CO_2 (Air Liquide, Portugal; to decrease pH) or by CO_2-filtered aeration (to increase pH) using air pumps (Stella 200, Aqua One Pro, Aqua Pacific UK Ltd, United Kingdom). Seawater total alkalinity was also measured in every tank on a weekly basis, following the protocol previously described elsewhere (Sarazin et al., 1999) and the combination of total alkalinity (AT) and pH was used to calculate carbonate system parameters (average values obtained for each treatment can be consulted in **Annex 4, Table A.4.2.**). After 30 days of acclimation, 9 fish were randomly sampled in order to determine the baseline levels of each biomarker analyzed, following the same procedures described in **sections 2.4** and **2.5** (average basal levels can be consulted in **Annex 4, Table A.4.3.**).

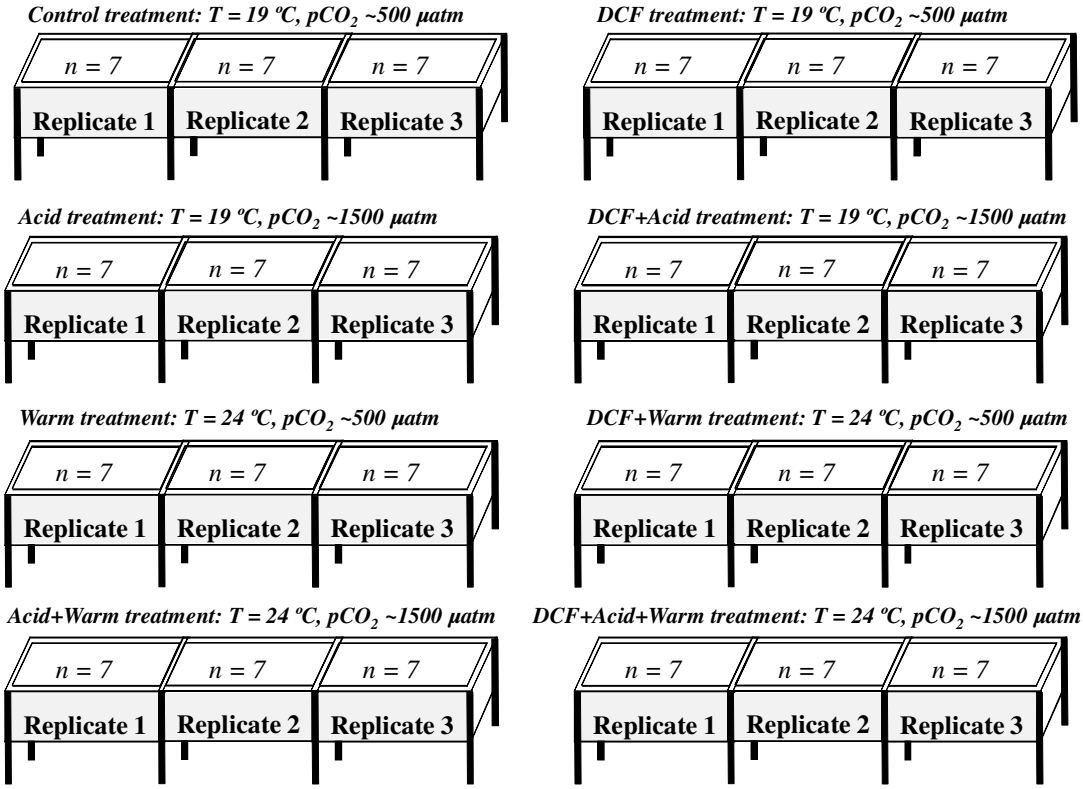

Figure 3.1.1. Experimental setup. Abbreviations: DCF – Diclofenac exposure; Acid – simulated acidification (i.e. pCO_2 ~1500 µatm, equivalent to pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC).

2.3. Exposure to DCF, warming and acidification

One week before initiating DCF exposure, seawater temperature and pCO_2 were slowly adjusted (+1 ºC and -0.1 pH unit per day), until reaching 24 ºC and ~1500 µatm pCO_2 (equivalent to pH = 7.6 units) in tanks simulating climate change conditions (i.e. treatments Acid, Warm, Acid+Warm, DCF+Acid, DCF+Warm and DCF+Acid+Warm; **Figure 3.1.1.**), according to the projections of the Intergovernmental Panel for Climate Change (scenario RCP8.5 of IPCC, 2014; McNeil and Sasse, 2016). The remaining seawater abiotic conditions (i.e. DO, salinity and photoperiod) were kept as previously described in **section 2.2.** (i.e. as during the acclimation period).

Eight treatments (4 non-contaminated and 4 contaminated; treatments randomly assigned to each tank) were carried out (n = 7 animals per replicate tank of treatment, i.e. a total of 21 animals per treatment; **Figure 3.1.1.**), simulating the reference temperature (i.e. 19 ºC) and pCO_2 (~500 µatm; 8.0 pH units) conditions, as well as the projected seawater warming (ΔT ºC = +5 ºC) and acidification (ΔpCO_2 ~1000 µatm; equivalent to ΔpH = -0.4 units), using a full cross-factorial design: i) Control treatment, i.e. fish daily fed with CTR feed (2% bw) and exposed to reference

temperature and pH conditions; ii) Acid treatment, i.e. fish daily fed with CTR feed (2% bw) and exposed to acidification (1500 µatm pCO_2, equivalent to pH = 7.6 units); iii) Warm treatment, i.e. fish daily fed with CTR feed (2% bw) and exposed to warming (24 °C); iv) Acid+Warm, i.e. fish daily fed with CTR feed (2% bw) and exposed to warming and acidification (24ºC and ~1500 µatm pCO_2); v) DCF treatment, i.e. fish daily fed with DCF-enriched feed (2% bw) and exposed to reference temperature and pH conditions; vi) DCF+Acid treatment, i.e. fish daily fed with DCF-enriched feed (2% bw) and exposed to acidification (1500 µatm pCO_2); vii) DCF+Warm treatment, i.e. fish daily fed with DCF-enriched feed (2% bw) and exposed to warming (24 ºC); viii) DCF+Acid+Warm treatment, i.e. fish daily fed with DCF-enriched feed (2% bw) and exposed to acidification and warming (24ºC and ~1500 µatm pCO_2). Seawater abiotic parameters were daily checked and adjusted to adequate levels whenever needed, as described above. No mortality was observed during the 28 days trial. Throughout the trial, seawater samples were collected from each tank (treatment; days 0, 7, 14 and 28 of the experiment), and DCF concentrations were determined according to the previously optimized and validated method of Cunha et al. (2017). Values were below the detection limit of the method (for water samples = 0.05µg L^{-1}) in all samples, thus, assuring that no external contamination was taking place in non-contaminated treatments, as well as, in DCF contaminated treatments (apart from the intended DCF dietary exposure through feed).

2.4. Samples preparation and haematological parameters

By the end of the exposure period, 9 fish were randomly collected from each treatment (i.e. 3 fish collected from each of the 3 replicate tanks that composed one treatment), euthanized by immersion in an overdosed MS222 solution (2000 mg L^{-1}; Sigma-Aldrich, USA) buffered with sodium bicarbonate (1 g of $NaHCO_3$ to 1 g of MS222 to 1 L of seawater) for 10 min. Euthanized fish were measured (total length, TL, and weight, W), and peripheral blood was collected by puncture of the caudal vein.

A fraction of fish blood was immediately used to perform the trypan blue exclusion test of cell viability previously described elsewhere (Strober, 2001). In brief, a 0.4% solution of trypan blue (trypan blue powder reagent, ~40% dye content, Sigma-Aldrich) was prepared in PBS (pH = 7.2 - 7.3 units) and then 100 µL of blood samples (proper diluted) were added to 100 µL trypan blue in 1.5 mL microtubes. Then, mixed sample was incubated (~ 3 min), loaded on a Neubauer hemocytometer (HBG, Germany) and analysed using an optical microscope (OPTIKA B-500, Italy) at a 400× magnification (three observations were made per individual; the average of the three measurements made in each sample/individual was used in statistical analysis).

Fish blood smears were also prepared on preclean glass microscopy slides (three slides per individual), allowed to air-dry and then fixed for 20 min in ethanol (100%) and stained with the

ready to use Hemacolor staining reagent (Hemacolor® Rapid staining of blood smear, Sigma-Aldrich) according to the instructions provided in this product, in order to subsequently count blood cells (erythrocytes and leukocytes), as well as to detect the presence of erythrocytes nuclear abnormalities (ENAs) and micronuclei, through optical microscopy. Following staining, the microscope glass slides were mounted with DPX (BDH, Poole, England). A minimum of 500 cells per slide were examined under the microscope (1000× magnification; the average of the three slide observations made in each sample/individual was used in statistical analysis). ENAs and micronuclei were counted according to the following the classification described in Carrasco et al. (1990).

After blood collection, fish were dissected, and fish muscle, brain and liver tissues were collected (approximately 100 mg of muscle and liver, and about 40 mg of brain), homogenized in ice-cold conditions with 1.0 mL of phosphate buffered saline (PBS; 140 mM NaCl, 3mM KCl, 10 mM KH_2PO_4, pH = 7.40 ± 0.02; reagents from Sigma-Aldrich, Germany), using an Ultra-Turrax® device (T25 digital, Ika, Germany). Crude homogenates were centrifuged in 1.5 mL microtubes for 15 minutes at 10.000 *g* and 4 ºC, supernatants were transferred to new microtubes, immediately frozen and kept at -80 ºC until further analyses. All biochemical analyses were performed in triplicate and using reagents of pro analysis grade or higher.

2.5. Molecular biomarkers

Eight molecular biomarkers (of exposure and/or effect) were selected to assess distinct biological effects (endpoints) induced by DCF, warming and acidification co-exposure, at the tissue level (**Table 3.1.1.**). **Table 3.1.1.** presents a summary of the selected biomarkers, as well as the different methodologies used. These molecular biomarkers have been widely used in ecotoxicological studies, being previously considered reliable and suitable to assess the effects of xenobiotics exposure (namely, DCF; e.g. Gonzalez-Rey and Bebianno, 2014), as well as of climate change-related effects (e.g. Rosa et al., 2016; Jesus et al., 2017; Maulvault et al., 2017). Thus, not only were they considered to fit the purposes of the present study, but also their use enabled comparisons of the present data with previous studies on the ecotoxicological effects of xenobiotics and/or climate change effects. Furthermore, to have a broader view of the effects of the studied stressors in a whole organism context, three different fish tissues (brain, liver and muscle) with distinct cell types and susceptibility to stressors were analysed.

Table 3.1.1. Summary of selected biomarkers and the corresponding methodologies used.

Biomarkers	Category	Tested ecotoxicological response	Type of methodology used	Methodology references
Animal condition (K), hepatosomatic index (HIS) and brain to body mass ratio (BB_{ratio})	Biomarker of effect	Animal fitness and/or disease	Morphometric assessment	Ricker (1975); Diniz et al. (2010)
Blood cell counts	Biomarker of effect	Immune capacity	Optical microscopy	Mummford et al. (2007)
Erythrocytes nuclear anomalies (ENAs)	Biomarker of effect	Genotoxicity	Optical microscopy	Carrasco et al. (1990)
Erythrocytes viability	Biomarker of effect	Citotoxicity	Optical microscopy	Strober (2001)
Catalase (CAT) activity	Biomarker of effect	Oxidative stress	Enzymatic assay	Johansson and Borg (1988); Maulvault et al. (2017)
Superoxide dismutase (SOD) activity	Biomarker of effect	Oxidative stress	Enzymatic assay	Sun et al. (1988); Maulvault et al. (2017)
Glutathione S-transferase (GST) activity	Biomarker of effect	Oxidative stress and xenobiotic detoxification phase II	Enzymatic assay	Habig et al. (1974); Maulvault et al. (2017)
Lipid peroxidation (LPO)	Biomarker of effect	Oxidative stress and cellular damage	TBARS method	Uchiyama and Mihara (1978); Madeira et al. (2016)
HSP70/HSC70 proteins content	Biomarker of effect	Chaperoning, heat shock response	Indirect ELISA	Njemini et al. (2005); Maulvault et al. (2017)
Total Ubiquitin (Ub) content	Biomarker of effect	Protein degradation and DNA repair	Direct ELISA	Madeira et al. (2014)
Acetylcholinesterase (AChE) activity	Biomarker of effect and exposure	Neurotoxicity	Enzymatic assay	Ellman et al. (1961); Maulvault et al. (2017)
Vitellogenin (VTG) content	Biomarker of exposure	Endocrine disruption and reproduction	Direct ELISA	Denslow et al. (1999); Diniz et al. (2010)

In order to normalize the results of each biomarker (i.e. results presented in mg of protein), total protein levels were also quantified in each sample according to the Bradford assay (Bradford, 1976). All protocols used for both enzymatic and protein quantification assays were adapted to 96-well microplates, being previously validated and described in detail in earlier reports (e.g. Diniz et al., 2010; Madeira et al., 2016a; Maulvault et al., 2017). In all methodologies, 96-well microplates from Nunc-Roskilde (Denmark) were used, as well as a microplate reader (BioRad, Benchmark, USA).

2.6. Animal fitness indexes (K, HSI and BB_{ratio}) and Integrated Biomarker Responses (IBR)

The Fulton's K index was directly calculated from the biometric data to determine fish condition, according to the formula:

$$K = 100\ x\ \frac{W\ (g)}{TL^3(cm)}$$

where W is the fish weight and TL is the total length (Ricker, 1975). The relationship between fish total weight and the respective organ weight was calculated for liver (i.e. hepatosomatic index, HSI) and brain (i.e. brain to body mass ratio, BB_{ratio}) to provide information on liver and brain condition, using the following equations:

$$HSI = \frac{Liver\ weight\ (g)}{W\ (g)}\ x\ 100$$

and,

$$BB_{ratio} = \frac{brain\ weight\ (g)}{W\ (g)}\ x\ 100$$

To better understand and relate the ecotoxicological effects induced by each experimental treatment, biomarkers related to animal fitness in a whole organism context (i.e. K, HSI, BB_{ratio} and haematological parameters), as well as molecular biomarkers in the different fish tissues (i.e. CAT, SOD, GST, LPO and AChE activities and HSP70/HSC70, Ub and VTG contents) were integrated by calculating the "Integrated biomarker response" (IBR) for each treatment (and tissue, for molecular biomarkers), according to the methodology proposed by Beliaeff and Burgeot (2002), later modified by Guerlet et al. (2010) (full details regarding the IBR methodology are available in these earlier reports, as well as, in Serafim et al., 2012, Ferreira et al., 2015; Madeira et al., 2016a). As IBR compares biomarker responses of organisms exposed to stressors to those of animals under control conditions (or their baseline levels), in general, lower biomarker scores (and, thus, lower IBR index values) are indicative of a better health state (higher

animal fitness), whereas higher scores usually indicate that organisms are in a poorer physiological condition (i.e. stressed; e.g. Serafim et al., 2012; Ferreira et al., 2015; Madeira et al., 2016a). Star plots and IBR calculations were performed using the Microsoft Excel software.

2.7. Statistical analysis

As standard procedure, data were first tested for normality and homoscedasticity through Kolmogorov–Smirnov and Levene tests, respectively. Data were log-transformed or square-rooted, whenever at least one of these assumptions of ANOVA was not verified. To evaluate the presence of significant differences between treatments in fish morphometric data (W and TL), haematological parameters (percentage of erythrocytes, leukocytes, ENAs, micronuclei and viable erythrocytes in relation to total blood cells or total erythrocyte counts), brain AChE activity and liver VTG content, one-way nested-ANOVA analysis was performed (with replicate tank as nesting factor), whereas for the remaining biochemical biomarkers (CAT, SOD, GST, LPO, HSP70/HSC70 and Ub), two-way ANOVAs were carried out instead, using tissue (brain, liver and muscle) and treatment (non-contaminated/DCF contamination, reference temperature/warming and reference pH/acidification) as variables. The existence of significant differences between whole organism and tissues IBR was also analysed using a simple one-way ANOVA. Finally, differences in animal fitness indexes were tested using an ANCOVA analysis, with W and TL as co-variates of K, and W as co-variate of HSI and BB_{ratio}. Post-hoc Tukey HSD tests were subsequently conducted to identify significant differences. Statistical analyses were performed at a significance level of 0.05, using STATISTICA™ software (Version 7.0, StatSoft Inc., USA).

3. Results

3.1. Fish morphometry and condition

Although higher TL and W were found in Control fish after 28 days of exposure compared to the baseline values, similar animal condition (K), hepatosomatic index (HSI) and brain to body mass ratio (BB_{ratio}) were observed (**Table 3.1.2.** and **Annex 4, Table A.4.3.***)*. Comparing with results observed under the reference temperature and pCO_2 conditions (Control treatment), warming promoted a significant increase in animal TL (**Table 3.1.2.**; $p = 0.02$). On the other hand, fish from DCF treatment exhibited significantly lower W than those exposed to warming and/or acidification (i.e. $p = 0.007$, $p = 0.002$, $p = 0.005$, $p = 0.021$ and $p = 0.039$ in Acid, Warm, Acid+Warm, DCF+Acid and DCF+Warm treatments, respectively), but not compared to Control treatment ($p = 0.115$; **Table 3.1.2.**). Regarding K, despite the general decrease promoted by the three stressors (alone or combined) compared to the Control treatment, significant differences were only observed when warming was combined with DCF, regardless of pCO_2 conditions

(corresponding to a 12% and 10% decrease in DCF+Warm and DCF+Acid+Warm, respectively, in relation to the Control treatment; $p = 0.006$ and $p = 0.024$, respectively; **Table 3.1.2.**, as well as **Annex 4, Table A.4.4.**). Overall, DCF, warming and/or acidification decreased fish liver size, with fish exposed to DCF alone or to the three stressors simultaneously (i.e. DCF+Acid+Warm treatment) exhibiting significantly lower HSI than non-contaminated samples, regardless of temperature and pCO_2 conditions (equivalent to a 55% and 34% decrease in relation to Control treatment, respectively; $p = 0.001$ in both cases; **Table 3.1.2.** and **Annex 4, Table A.4.4.**). Interestingly, DCF alone also yielded significantly lower HSI than DCF and acidification co-exposure (i.e. treatment DCF+Acid; $p = 0.005$; **Table 3.1.2.**). All stressors acting alone or in combination promoted a significant reduction of the BB_{ratio} (equivalent to a decrease of 18% to 47% in DCF+Acid+Warm and Acid treatments, respectively, compared to the Control; $p = 0.003$ and $p = 0.001$, respectively; **Table 3.1.2.** and **Annex 4, Table A.4.4.**). In addition, non-contaminated fish exposed to acidification (at both temperature exposures; i.e. Acid and Acid+Warm treatments) also revealed significantly lower BB_{ratio} than non-contaminated fish exposed to warming alone (i.e. Warm treatment; $p = 0.008$; **Table 3.1.2.**).

Table 3.1.2. Total length (TL; cm) and weight (W; g), Fulton's condition index (K), hepatosomatic index (HIS) and brain-to-body mass ratio (BB_{ratio}) in *D. labrax* after 28 days of exposure (mean ± SD; $n = 9$). In each column, different letters indicate significant differences ($p < 0.05$) between treatments. Abbreviations: Control – reference temperature and pH conditions (i.e. T = 19 ºC and pH = 8.0 units); DCF – DCF exposure; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC).

	TL	W	K	HSI	BB_{ratio}
Control	7.7 ± 0.2^{bc}	8.9 ± 0.7^{ab}	1.94 ± 0.10^{a}	2.41 ± 0.18^{a}	1.05 ± 0.08^{a}
Acid	8.2 ± 0.2^{ab}	10.1 ± 0.6^{a}	1.86 ± 0.01^{ab}	1.97 ± 0.32^{ab}	0.56 ± 0.05^{c}
Warm	8.4 ± 0.2^{a}	10.7 ± 1.5^{a}	1.78 ± 0.15^{abc}	2.17 ± 0.58^{ab}	0.78 ± 0.04^{b}
Acid+Warm	8.2 ± 0.5^{ab}	10.3 ± 1.2^{a}	1.88 ± 0.15^{a}	2.03 ± 0.33^{ab}	0.59 ± 0.01^{c}
DCF	7.3 ± 0.6^{c}	7.3 ± 1.5^{b}	1.87 ± 0.11^{a}	1.08 ± 0.42^{c}	0.79 ± 0.13^{b}
DCF+Acid	8.1 ± 0.4^{ab}	9.7 ± 1.2^{a}	1.82 ± 0.01^{abc}	2.28 ± 0.72^{ab}	0.74 ± 0.11^{b}
DCF+Warm	8.2 ± 0.3^{ab}	9.4 ± 0.8^{a}	1.70 ± 0.07^{c}	1.70 ± 0.53^{abc}	0.75 ± 0.12^{b}
DCF+Acid+Warm	8.0 ± 0.3^{ab}	9.0 ± 1.0^{ab}	1.75 ± 0.02^{bc}	1.60 ± 0.19^{bc}	0.86 ± 0.04^{b}

3.2. Haematological parameters

Similar haematological parameters were found in the baseline and Control groups (**Table 3.1.3.** and **Annex 4, Table A.4.3.**). Significantly different percentages of erythrocytes (lower; $p = 0.047$) and leukocytes (higher; $p = 0.046$) were found in non-contaminated fish under acidification and warming co-exposure compared to the remaining treatments, with the exception of DCF+Warm treatment ($p > 0.050$; **Table 3.1.3.**). DCF exposed fish exhibited significantly

higher percentages of erythrocytes nuclear abnormalities (ENAs) compared to non-contaminated fish, regardless of temperature and pCO_2 levels (between 27.1% and 33.7% in DCF+Acid+Warm and DCF treatments, respectively; $p = 0.040$ and $p = 0.002$, respectively; **Table 3.1.3.** and **Annex 4, Table A.4.3.**). Noteworthy, in general, no micronuclei (i.e. the most severe form of ENA) were observed in non-contaminated fish (except in two samples of the Acid treatment, corresponding to an average of 1.5%), whereas up to 12.5% of micronuclei where found in contaminated fish (i.e. maximum number of micronuclei observed in treatment DCF; $p < 0.0001$; **Table 3.1.3.**). Similarly, erythrocytes' viability was reduced by all stressors, except warming acting alone ($p > 0.050$), yet, the lowest values were observed in fish under the co-exposure of DCF and acidification, regardless of temperature, (averages ranging from 32.9% to 39.8%; $p = 0.001$ in both cases; **Table 3.1.3.**).

Table 3.1.3. Percentage of erythrocytes and leukocytes in relation to total cell counts, as well as percentage of viable erythrocytes (Viable Ery), erythrocyte nuclear abnormalities (ENAs) and micronuclei in relation to total erythrocyte counts (mean ± SD; $n = 9$). In each column, different letters indicate significant differences ($p < 0.05$) between treatments. Abbreviations: Control – reference temperature and pH conditions (i.e. T = 19 ºC and pH = 8.0 units); DCF – DCF exposure; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC).

	Erythrocytes	Leucocytes	ENAs	Micronuclei	Viable Ery
Control	95.6 ± 1.2^{a}	4.4 ± 1.2^{b}	11.5 ± 6.8^{b}	0.0 ± 0.0^{c}	76.2 ± 3.4^{a}
Acid	98.2 ± 0.7^{a}	1.8 ± 0.7^{b}	9.5 ± 1.7^{b}	1.5 ± 2.6^{bc}	64.5 ± 2.6^{b}
Warm	96.9 ± 1.1^{a}	3.1 ± 1.1^{b}	12.1 ± 3.9^{b}	0.0 ± 0.0^{c}	76.4 ± 2.5^{a}
Acid+Warm	90.3 ± 4.5^{b}	9.7 ± 4.5^{a}	15.1 ± 1.9^{b}	0.0 ± 0.0^{c}	68.0 ± 3.0^{b}
DCF	97.0 ± 0.5^{a}	3.0 ± 0.5^{b}	33.7 ± 4.9^{a}	8.0 ± 4.0^{a}	47.7 ± 2.0^{c}
DCF+Acid	98.2 ± 0.9^{a}	1.8 ± 0.9^{b}	31.3 ± 7.7^{a}	5.0 ± 4.4^{ab}	39.8 ± 3.3^{de}
DCF+Warm	95.4 ± 0.6^{ab}	4.6 ± 0.6^{ab}	30.3 ± 5.8^{a}	4.9 ± 3.9^{ab}	45.4 ± 5.9^{cd}
DCF+Acid+Warm	97.4 ± 1.5^{a}	2.6 ± 1.5^{b}	27.1 ± 6.4^{a}	5.9 ± 5.2^{ab}	32.9 ± 2.2^{e}

3.3. Antioxidant defences and lipid peroxidation

Basal antioxidant biomarker levels were similar to those in the Control treatment (**Figures 3.1.2.A-C** and **Annex 4, Table A.4.3.**). Overall, different patterns were observed according to tissue and treatment. CAT activity significantly varied among tissues of contaminated fish (being higher in the liver followed by brain and muscle; $p < 0.050$), but not in non-contaminated tissues (**Figures 3.1.2.A-C**). In fish muscle, DCF exposure inhibited CAT's activity (between 57 and 80%, in DCF+Warm and DCF treatments, respectively; $p = 0.046$ and $p = 0.007$, respectively), regardless of temperature and pCO_2 exposure **Figure 3.1.2.A**, as well as **Annex 4, Table A.4.4.**). In liver, although values in Control treatment were not significantly different from those in the

remaining treatments, DCF exposure alone resulted in significantly higher CAT activity than when combined with acidification ($p = 0.001$ and $p = 0.047$ in DCF+Acid and DCF+Acid+Warm treatments, respectively; **Figure 3.1.2.B**). As for the brain, no significant differences were found between treatments ($p > 0.050$; **Figure 3.1.2.C**).

Concerning SOD, higher inhibition was observed in fish brain and liver (between 63.1% and 78.5%) compared to muscle (between 39.3% and 72.1%), regardless of treatment ($p < 0.05$; **Figures 3.1.2.D-F**). In fish muscle, DCF exposure, warming and acidification drastically increased SOD inhibition, with the highest values being observed in non-contaminated acidified treatments, corresponding to an average increase of 58% to 67% in relation to the Control treatment ($p < 0.0001$ in Acid and Acid+Warm treatments; **Figure 3.1.2.D**, as well as **Annex 4, Table A.4.4.**). Noteworthy, higher SOD inhibition percentages were found in non-contaminated muscle ($p < 0.0001$; **Figure 3.1.2.D**). A different trend was observed in the liver, as fish exposed to acidification and DCF (alone or combined) showed significantly lower SOD inhibition than Control fish (i.e. a reduction of 9-11%; $p < 0.0001$ in Acid, DCF and DCF+Acid treatments; **Figure 3.1.2.E**, as well as **Annex 4, Table A.4.4.**). As for fish brain, overall, SOD activity did not vary among treatments, with the exception of DCF exposure combined with acidification (i.e. 70.3% of SOD inhibition), which yielded significantly lower inhibition compared to DCF exposure alone (i.e. 75.8% of SOD inhibition; $p = 0.021$; **Figure 3.1.2.F**).

Overall, fish liver exhibited higher GST activity than the muscle and brain, with values ranging between 19.0 and 51.7 nmol min^{-1} mg $protein^{-1}$ in this tissue ($p < 0.050$; **Figures 3.1.2.G-I**). In fish muscle, DCF exposure combined with warming resulted in significantly higher GST activity compared to the other treatments (corresponding to an increase of 88% in relation to the Control treatment; $p < 0.0001$; **Figure 3.1.2.G**, as well as **Annex 4, Table A.4.4.**). Conversely, acidification induced the opposite effect, resulting in an average reduction of 33% in relation to the Control treatment ($p = 0.022$; **Figure 3.1.2.G**, as well as **Annex 4, Table A.4.4.**). A similar trend was found in fish liver, with Acid treatment also evidencing an impairment of GST activity in relation to the Control treatment (i.e. 24% of average reduction; $p = 0.008$), yet, warming alone was responsible for the highest induction of this enzyme (corresponding to an increase of 63% in Warm treatment in relation to the Control treatment; $p = 0.012$; **Figure 3.1.2.H**, as well as **Annex 4, Table A.4.4.**). In the brain, with the exception of Acid treatment, all treatments induced GST activity compared to Control conditions, but the highest induction was observed when the three stressors were combined (i.e. in DCF+Acid+Warm treatment; $p < 0.0001$; **Figure 3.1.2.I**, as well as **Annex 4, Table A.4.4.**).

Lipid peroxidation (measured as MDA concentration) in tissues from each treatment is shown in **Figure 3.1.3**. In general, higher LPO was observed in brain and muscle compared to liver ($p < 0.050$; **Figure 3.1.3.**). As for differences among treatments, in muscle, significantly higher LPO

was observed in non-contaminated fish under warmer conditions (p = 0.009 in Warm and p < 0.0001 Acid+Warm treatments, respectively), regardless of pCO_2 levels, as well as in DCF+Warm treatment (p = 0.023; **Figure 3.1.3.A** and **Annex 4, Table A.4.4.**). On the other hand, DCF exposure combined with acidification (regardless of temperature) yielded lower LPO values than those observed in the Control treatment (equivalent to an average decrease of 30% and 48% in DCF+Acid and DCF+Acid+Warm treatments, respectively; p = 0.027 and p = 0.030, respectively; **Figure 3.1.3.A** and **Annex 4, Table A.4.4.**). In liver and brain, none of the treatments simulating DCF exposure, acidification and/or warming was significantly different from the Control treatment (**Figures 3.1.3.B**,**C**). However, in fish brain, significantly higher LPO was observed in Acid+Warm and DCF treatments compared to treatments simulating the stressors co-exposure (i.e. p = 0.042, p = 0.034 and p = 0.018 in DCF+Acid, DCF+Warm and DCF+Acid+Warm treatments, respectively; **Figure 3.1.3.C**).

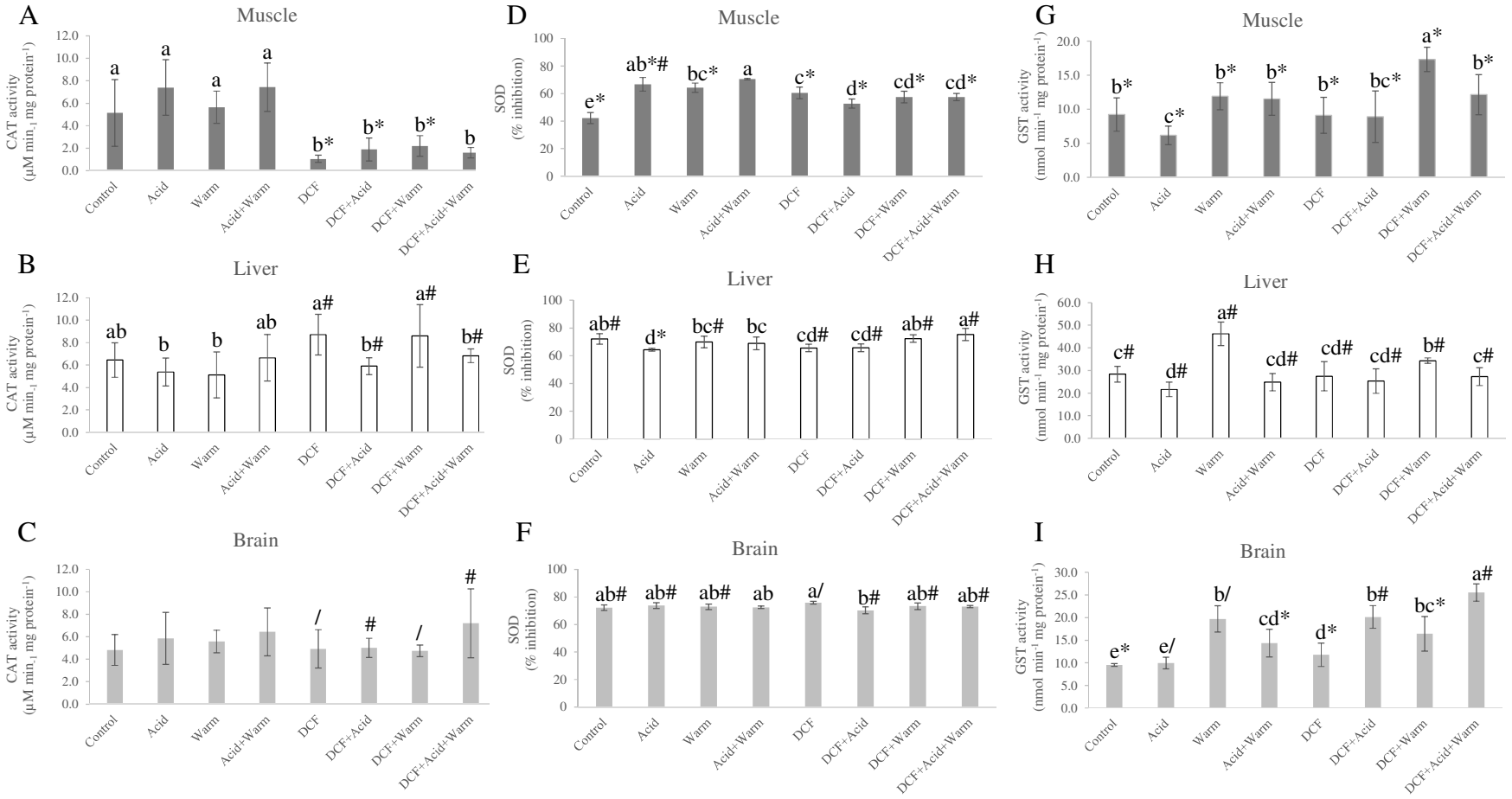

Figure 3.1.2. Antioxidant enzymes activity (GST, nmol min $^{-1}$ mg^{-1} protein; CAT, µM min $^{-1}$ mg^{-1} protein; SOD, % inhibition) in muscle (A, D, G), liver (B, E, H) and brain (C, F, I) of *D. labrax* after 28 days of exposure (mean ± SD; n = 9). Different letters indicate significant differences between treatments (p < 0.05), whereas different symbols (*, # and /) indicate significant differences between tissues in the same treatment. Abbreviations: Control – reference temperature and pH conditions (i.e. T = 19 ºC and pH = 8.0 units); DCF – DCF exposure; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC), CAT – catalase activity; SOD – superoxide dismutase inhibition; GST – glutathione S-transferases.

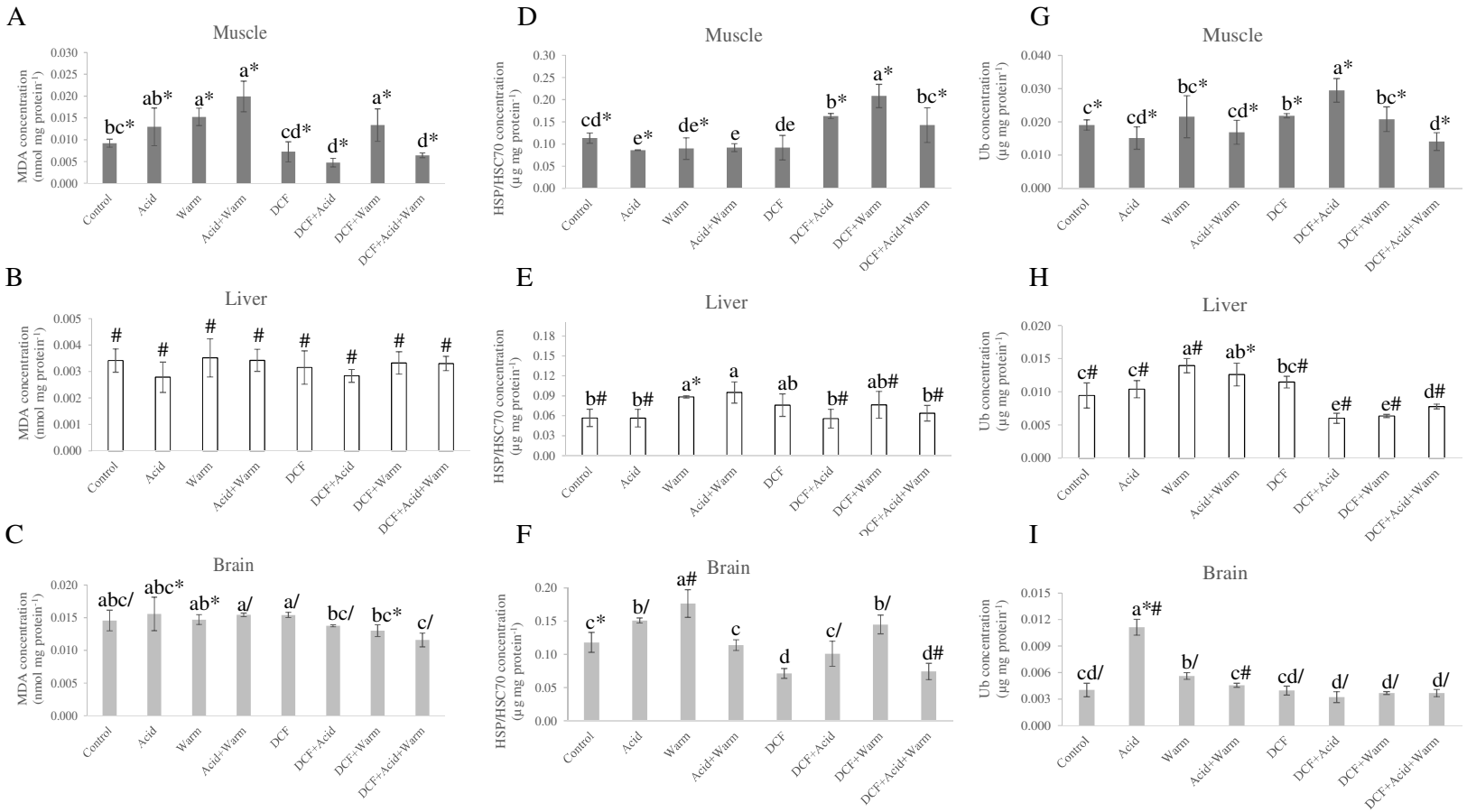

Figure 3.1.3. Lipid peroxidation (expressed as MDA concentration; nmol mg^{-1} protein), HSP/HSC70 concentration (µg mg^{-1} protein), and ubiquitin concentration (Ub; µg mg^{-1} protein) in muscle (A, D, G), liver (B, E, H) and brain (C, F, I) of *D. labrax* after 28 days of exposure (mean ± SD; $n = 9$). Different letters indicate significant differences between treatments ($p < 0.05$), whereas different symbols (*, # and /) indicate significant differences between tissues in the same treatment. Abbreviations: Control – reference temperature and pH conditions (i.e. T = 19 ºC and pH = 8.0 units); DCF – DCF exposure; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC); MDA –lipid peroxidation measured as malondialdehyde concentration; HSP70/HSC70 – heat shock proteins; Ub – total ubiquitin.

3.4. Chaperoning and protein degradation

Basal HSP70/HSC70 and Ub protein contents were similar to those in the Control treatment (**Figures 3.1.3.D-I**, and **Annex 4, Table A.4.3.***)*. In general, higher concentrations of HSP70/HSC70 proteins were found in fish muscle and brain compared to liver ($p < 0.050$; **Figures 3.1.3.D-F**). In comparison with the Control treatment, significantly higher levels of these proteins were observed in the muscle of contaminated fish under acidification or warming alone (i.e. an average increase of 44% and 84% in treatments DCF+Acid and DCF+Warm, respectively; $p = 0.033$ and $p < 0.0001$, respectively; **Figure 3.1.3.D Annex 4, Table A.4.4.**). In contrast, HSP70/HSC70 proteins synthesis in the muscle was impaired under acidification alone (i.e. an average reduction of 24% in Acid treatment in relation to the Control treatment; $p = 0.038$; **Figure 3.1.3.D** and **Annex 4, Table A.4.4.**). Increased temperatures resulted in significantly higher HSP70/HSC70 protein concentrations in the liver of non-contaminated fish, but not in DCF exposed ones (equivalent to a 56% and 67% increase in Warm and Acid+Warm treatments, respectively; $p = 0.019$ and $p = 0.006$, respectively; **Figure 3.1.3.E** and **Annex 4, Table A.4.4.**). A different pattern was observed in fish brain, with HSP70/HSC70 protein concentrations being significantly higher in Acid, Warm and DCF+Warm treatments compared to the Control treatment (i.e. average increases of 28%, 50% and 33% in relation to Control, respectively; $p = 0.043$, $p = 0.009$ and $p = 0.047$, respectively), but significantly lower in DCF and DCF+Acid+Warm treatments (i.e. average decrease of 40%; $p = 0.012$ and $p = 0.025$, respectively; **Figure 3.1.3.F** and **Annex 4, Table A.4.4.**).

In general, higher Ub concentration was found in fish muscle compared to the liver and brain ($p < 0.050$; **Figures 3.1.3.G-I**). In comparison with the Control treatment, DCF alone or in co-exposure with acidification promoted significantly higher Ub concentrations in the muscle (i.e. an average synthesis induction of 15% and 55% in DCF and DCF+Acid treatments, respectively; $p = 0.041$ and $p = 0.006$, respectively; **Figure 3.1.3.G** and **Annex 4, Table A.4.4.**). Conversely, the combination of the three stressors inhibited the synthesis of Ub in the muscle, resulting in an average decrease of 26% in relation to the Control treatment (i.e. in DCF+Acid+Warm treatment; $p = 0.022$; **Figure 3.1.3.G** and **Annex 4, Table A.4.4.**). In liver, while warming (with or without acidification) enhanced Ub concentration (48% and 34% increase in relation to Control treatment; $p = 0.008$ and $p = 0.020$, respectively), DCF exposure combined with acidification and/or warming elicited the opposite effect (a reduction of 36%, 33% and 18% in DCF+Acid, DCF+Warm and DCF+Acid+Warm treatments, respectively; $p = 0.025$, $p = 0.039$ and $p = 0.043$, respectively; **Figure 3.1.3.H** and **Annex 4, Table A.4.4.**). As for fish brain, acidification and warming acting alone increased Ub concentration (i.e. average increase of 40% and over 100% in Warm and Acid treatments, respectively, compared to the Control treatment; $p = 0.007$ and $p < 0.0001$, respectively), while the remaining treatments concentrations did not significantly

differed from those observed in the Control treatment ($p > 0.050$; **Figure 3.1.3.I** and **Annex 4, Table A.4.4.**).

3.5. Neurotoxicity and endocrine disruption

Basal AChE brain activity and VTG content were similar to those in the Control treatment (**Figure 3.1.4.** and **Annex 4, Table A.4.3.***)*. In non-contaminated fish, warming acting alone revealed a significantly higher AChE activity (i.e. in Warm treatment) compared to treatments simulating acidification (regardless of temperature exposure; $p = 0.041$ and $p = 0.031$ in Acid and Acid+Warm treatments, respectively), but not compared to the Control treatment **Figure 3.1.4.A**). In DCF exposed fish, increased AChE activity was observed under the reference temperature and pCO_2 conditions (i.e. in DCF treatment), or when the three stressors were acting together (i.e. in DCF+Acid+Warm), compared to acidification and warming acting alone (i.e. $p = 0.033$ and $p = 0.015$ in DCF+Acid and DCF+Warm treatments respectively; **Figure 3.1.4.A**). Noteworthy, DCF and DCF+Warm treatments were also significantly different from the Control treatment (17% higher and 13% lower, respectively, $p = 0.033$ and $p = 0.041$, respectively; **Figure 3.1.4.A** and **Annex 4, Table A.4.4.**).

Concerning VTG content in liver, fish exposed to warming (regardless of pCO_2 levels and DCF) generally revealed significantly higher VTG content than those exposed to the control temperature (i.e. $p = 0.013$, $p = 0.016$ and $p = 0.033$ in Warm, Acid+Warm, and DCF+Warm, respectively), whereas significantly lower values were observed under increased pCO_2 levels ($p > 0.050$; **Figure 3.1.4.B** and **Annex 4, Table A.4.4.**). It is also worth noting that DCF exposure acting alone also significantly enhanced VTG concentration in relation to the levels found in the Control treatment (equivalent to 87% of increase in DCF treatment; $p < 0.0001$; **Figure 3.1.4.B** and **Annex 4, Table A.4.4.**).

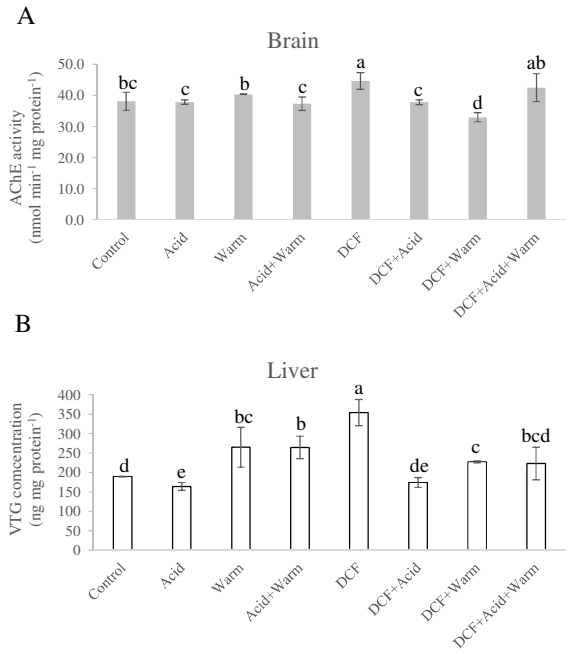

Figure 3.1.4. Acetylcholinesterase (AChE) activity (nmol min $^{-1}$ mg^{-1} protein; A) in brain tissues and vitellogenin (VTG) concentration (ng mg^{-1} protein; B) in liver tissues of *D. labrax* after 28 days of exposure (mean ± SD; n = 9). Different letters indicate significant differences between treatments ($p < 0.05$). Abbreviations: Control – reference temperature and pH conditions (i.e. T = 19 ºC and pH = 8.0 units); DCF – DCF exposure; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC); AChE – acetylcholinesterase activity; VTG – vitellogenin content.

3.6. Integrated Biomarker Responses (IBRs)

The starplots combining IBRs of the different biomarkers are presented in **Figure 3.1.5.** (individual biomarker scores can also be consulted in **Annex 4, Table A.4.5.**). Fish liver and brain were more responsive than muscle and whole organism (all treatments combined; One-way ANOVA, F = 6.1126; p = 0.0025; **Figure 3.1.5.A**). Furthermore, the different treatments promoted distinct biomarker responses in the whole organism, muscle, liver and brain levels (**Figure 3.1.5.** and **Annex 4, Table A.4.5.**). In terms of biomarkers assessed from a whole organism perspective, the combination of DCF exposure with climate change effects, particularly acidification, yielded the highest IBR index value with haematological parameters (erythrocytes:leukocytes total counts ratio, % ENAs and viable erythrocytes) being the most responsive biomarkers (i.e. highest scores), whereas the Control treatment resulted in the lowest (**Figures 3.1.5.A-E** and **Annex 4, Table A.4.5.**). Control treatment also resulted in the lowest IBR index value in muscle and brain, but not in liver (**Figures 3.1.5.A**,**F-Q** and **Annex 4, Table A.4.5.**). In fish muscle, the highest IBR was attributed to the combination of DCF exposure and warming, with higher biomarker scores obtained for SOD, GST and HSP70/HSC70 in this

treatment (**Figures 3.1.5.A,F-I** and **Annex 4, Table A.4.5.**). Warming alone resulted in the highest IBR index value in the liver, with GST, HSP70/HSC70 and Ub yielding the most contributing scores (**Figures 3.1.5.A,J-M** and **Annex 4, Table A.4.5.**). Noteworthy, acidification (regardless of DCF exposure) revealed the lowest IBR index values in fish liver (**Figures 3.1.5.A,J-M** and **Annex 4, Table A.4.5.**). In the brain, DCF exposure alone showed the highest IBR index value, predominated by the scores of CAT, SOD and AChE activities (**Figures 3.1.5.A,N-Q** and **Annex 4, Table A.4.5.**).

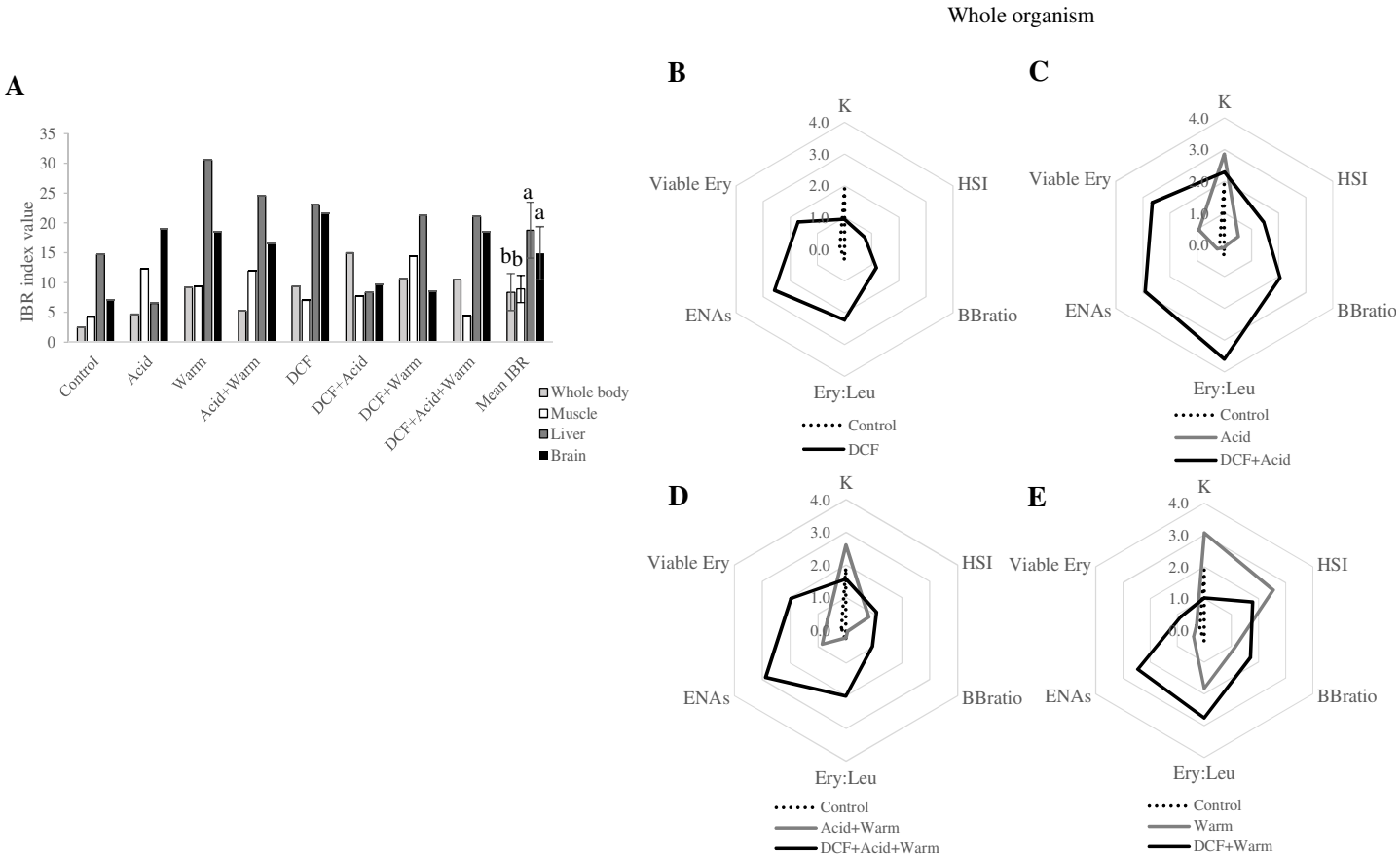

Figure 3.1.5. Total IBR index value and mean IBR (± standard deviation; all treatments combined; A), as well as, star plots for each treatment, including the different biomarkers analysed in the whole organism (B – E) and in the different fish tissues (muscle: F – I; liver: J– M; brain: N – Q). In **Figure 3.1.5.A**, different letters indicate significant differences in mean IBR ($p < 0.05$).

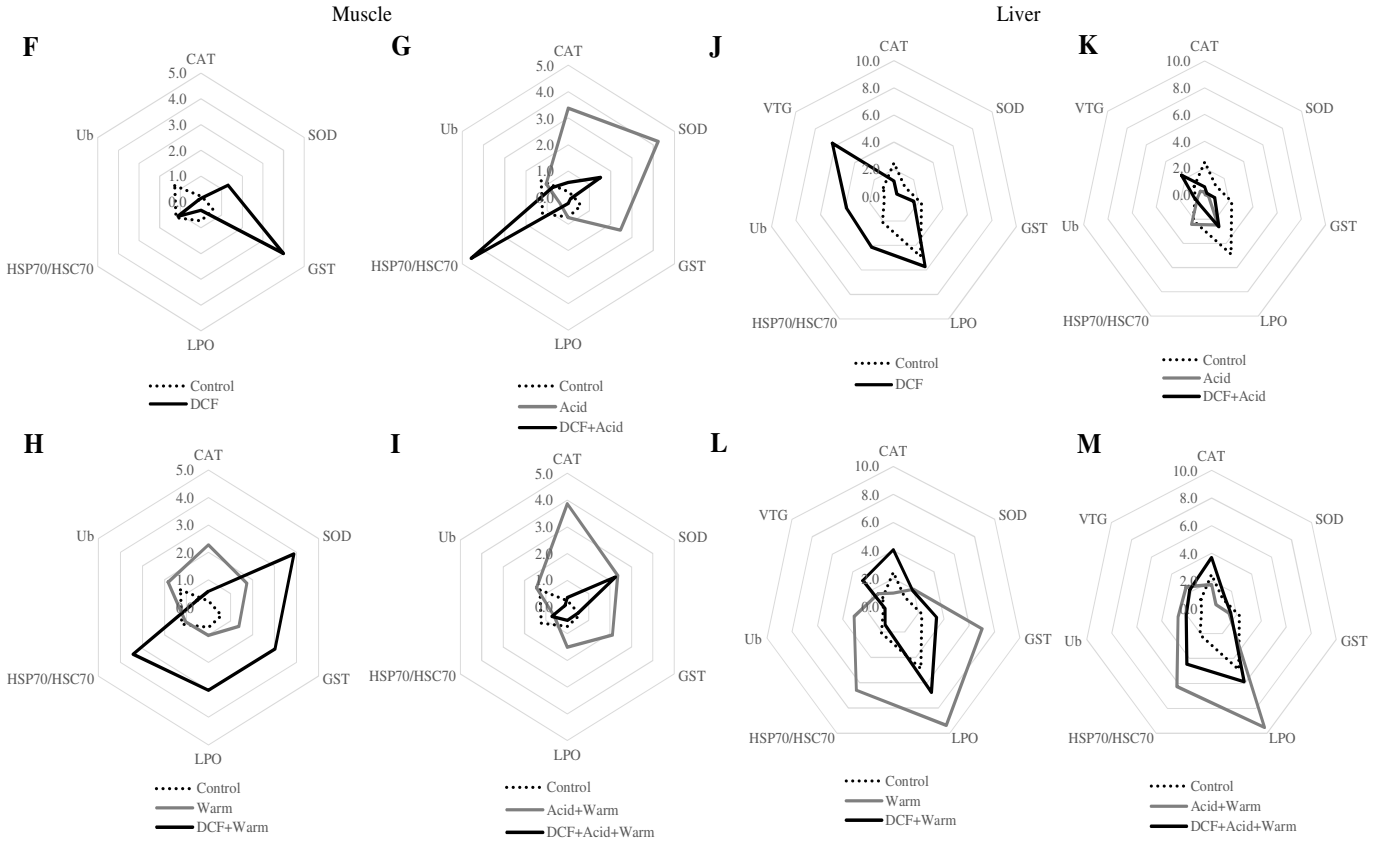

Figure 3.1.5. ***(continuation)*** Total IBR index value and mean IBR (± standard deviation; all treatments combined; A), as well as, star plots for each treatment, including the different biomarkers analysed in the whole organism (B – E) and in the different fish tissues (muscle: F – I; liver: J– M; brain: N – Q). In **Figure 3.1.5.A**, different letters indicate significant differences in mean IBR ($p < 0.05$).

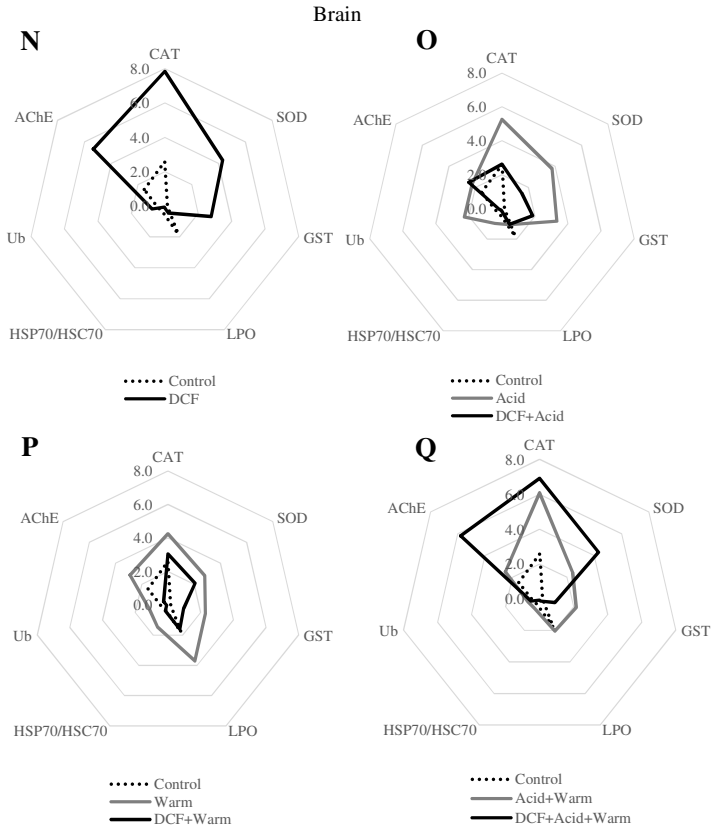

Figure 3.1.5. ***(continuation)*** Total IBR index value and mean IBR (± standard deviation; all treatments combined; A), as well as, star plots for each treatment, including the different biomarkers analysed in the whole organism (B – E) and in the different fish tissues (muscle: F – I; liver: J– M; brain: N – Q). In **Figure 3.1.5.A**, different letters indicate significant differences in mean IBR ($p < 0.05$). Abbreviations: K - Fulton's condition index; HIS - hepatosomatic index; BB_{ratio} - brain-to-body mass ratio, Ery:Leu – ratio between total erythrocytes and total leukocytes counts; ENAs - erythrocytes nuclear abnormalities (including micronuclei); Viable Ery - viable erythrocytes; CAT – catalase; SOD – superoxide dismutase; GST – glutathione S-transferases; LPO - lipid peroxidation; HSP70/HSC70 - heat shock proteins; Ub - total ubiquitin; AChE – acetylcholinesterase; VTG – vitellogenin; DCF – DCF exposure; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC).

4. Discussion

Studies assessing the interactions between chemical contaminants and climate change-related stressors are still very limited, both in field or laboratory conditions. Yet, the distinct trends observed according to treatment, biomarker and tissue highlighted the importance, not only of analysing different biomarkers/tissues to have a broader view of stressors in a whole organism context, but also of considering the potential interactions between multiple stressors in ecotoxicological studies.

4.1. General animal fitness

As observed in the present study (i.e. higher TL in Warm treatment), warmer temperatures are usually associated to an enhanced animal growth (e.g. Maulvault et al., 2017; Sandblom et al., 2016). However, animal fitness can also gradually decline when temperature is beyond species optimal temperature range or when additional stressors take place concomitantly, implying additional energetic costs (Pörtner and Peck, 2010) or preventing the allocation of energy resources towards somatic growth (Pistevos et al., 2015; Sandblom et al., 2016; Maulvault et al., 2017; Anacleto et al., 2018), thus, justifying the diminished animal condition (i.e. lower K) observed when warming was combined with DCF exposure and acidification. Despite the exposure to chemical contaminants has been frequently linked to liver hypertrophy and/or hyperplasia (Diniz et al., 2009; Sadekarpawar and Parikh, 2013; Maulvault et al., 2017), a different trend was observed in this study, as DCF exposure alone or the combination of the three studied stressors resulted in an HSI reduction. A similar result was also reported for *Hoplias malabaricus* exposed to DCF from dietary sources (Guiloski et al., 2015). Such reduction could be due to: i) tissue damage induced by DCF (Triebskorn et al., 2004; Pandey et al., 2017), which could have been reversed by warming and acidification acting separately (i.e. DCF+Acid and DCF+Warm); and/or ii) severe metabolic changes induced by the combination DCF, warming and acidification (justifying the lower K registered in this treatment), which could have interfered with the deposition of glycogen in the liver and with the stability of structural components, such as lipids and proteins, thus, affecting liver's weight (Triebskorn et al., 2004; Anacleto et al., 2018).

The three stressors substantially decreased brain mass in relation to body weight, probably due to a reduction in the number of brain cells (i.e. cell death elicited by these stressors), which can likely result in cognitive impairments. Acidification (alone or in co-exposure with warming) led to the greatest brain mass reduction, and such result could be linked to CO_2 acid-base balance disturbances and to the osmoregulatory mechanisms that are triggered under increased pCO_2 levels (Nilsson et al., 2012; Heuer et al., 2016). Despite research on fish neurophysiology and the potential links with environmental stressors is still on its infancy, recent studies have demonstrated that both pollutants and climate change-related stressors (particularly, acidification)

can substantially alter fish behavior and cognition and, therefore, lead to dramatic ecological consequences (Nilsson et al., 2012; Lopes et al., 2016; Heuer et al., 2016; Maulvault et al., 2018).

4.2. Immune capacity and genotoxicity

Fish immune system can be either stimulated or suppressed by the exposure to xenobiotics (e.g. Bado-Nilles et al. 2009; Ribas et al., 2016), and hostile abiotic conditions can further immuno-compromise fish health (Chiaramonte et al., 2016). Moreover, the immunological and health-state changes induced by the environmental stressors have been previously associated to increased fish susceptibility to disease and, ultimately, to the decline of fish populations in the wild (e.g. Kennedy and Farrell, 2008; Sueiro and Palacios, 2016). Yet, contrasting the results reported by Saravanan et al. (2011), Ribas et al. (2016) and Mathias et al. (2018) in freshwater fish species *Cyprinus carpio*, *Hoplias malabaricus* and *Rhamdia quelen*, respectively, no significant alterations in erythrocytes and leukocytes counts were elicited by DCF in the present study. This could be related to a dose-dependent action of NSAIDs, such as DCF, or to distinct compound bioavailability according to the uptake pathway (i.e. water exposure in Saravanan et al., 2011; or trophic transfer through the ingestion of preys previously injected with DCF in Ribas et al., 2016). As for the effect of abiotic stressors, despite the stress induced by the combination of warming and acidification (i.e. Acid+Warm treatment) resulted in increased leukocyte counts, such immunostimulation, seemed to have been reversed by DCF (i.e. DCF+Acid+Warm treatment), most likely due to the anti-inflammatory action of this compound. In this way, while temperature changes have been previously described to have a suppressive effect on fish immune system (Magnadottir, 2006), further research is needed in what concerns the effects of seawater pH levels and the interactive effects with other stressors on fish immunity.

All stressors, except warming acting solely, significantly decreased erythrocytes' viability, and this result not only evidenced DCF's cytotoxicity, but also suggested that DCF's deleterious effects on fish immune capacity were enhanced by warming and acidification. Moreover, the frequency of observed ENAs, particularly, in its most severe form (i.e. micronuclei) was substantially increased by DCF exposure, regardless of temperature and pCO_2 conditions, therefore, evidencing DCF's genotoxic effects. Research on the immunological and genotoxicological effects of NSAIDs in marine fish species is extremely limited and, to the best of the authors' knowledge, the interactive effects of these compounds with abiotic variables have not been previously considered, thus, hindering comparisons with the current data. Nonetheless, recent studies on mollusks (e.g. *Dreissena polymorpha,* Parolini et al., 2011; and *Lymnaea stagnalis,* Boisseaux et al., 2017) did not find remarkable cyto-genotoxic effects following short-term exposure to DCF. Similarly, Guiloski et al. (2017) revealed no significant differences in the number of micronuclei between control and DCF exposed groups in the freshwater fish *Rhamdia*

quelen. As argued by Boisseaux et al. (2017), such absence of cyto-genotoxic effects in these studies may be due to different reasons, such as the exposure duration (short-term exposures were carried out in Parolini et al., 2011 and Boisseaux et al., 2017), contaminant concentration or exposure pathway (DCF exposure through water in Parolini et al., 2011, Boisseaux et al., 2017 and Guiloski et al. 2017), therefore, calling for the need to further investigate the influence of these aspects on DCF's modes of action.

4.3. Molecular responses

Results highlighted the importance of assessing the differential responses of fish tissues in ecotoxicological studies, providing a broader view on the impacts of environmental stressors, because different tissues not only had distinct baseline levels of molecular biomarkers, but also responded differently to DCF, warming and/or acidification co-exposure. In agreement with previous findings (e.g. Islas-Flores et al., 2013; Madeira et al., 2016a,b; Maulvault et al., 2017), the present data evidenced both up- and down biomarker regulations. Such distinct patterns were likely linked to the fact that the exposure to environmental stressors usually activates the defence and scavenging cell mechanisms (regardless of tissue), in order to overcome/adjust to the stress induced but, still, the opposite strategy (i.e. inhibition) can also occur when the stressor's severity and duration leads these biological mechanisms to exhaustion (Ferreira et al., 2015; Madeira et al., 2016a).

4.3.1. Antioxidant defences

In agreement with previous findings (Islas-Flores et al., 2013; Stepanova et al., 2013; Guiloski et al., 2015), the present results showed that DCF exposure (alone) affected fish antioxidant machinery in a tissue and biomarker specific way, for instance, significantly inhibiting CAT and SOD activity in the muscle (but not in the liver and brain) or enhancing GST activity in the brain (but not in muscle and liver). As for the interactive effects of temperature and pCO_2 levels, previous studies have evidenced how these two stressors can strongly mediate fish antioxidant machinery (e.g. Rosa et al., 2016; Maulvault et al., 2017), generally leading to increased CAT, SOD and GST activities, which matches some of the current findings (e.g. increased SOD activity in fish liver under acidification regardless of DCF exposure, as well as increased GST activity in all tissues under warming and in muscle and brain of contaminated fish under acidification), but not all. First, 5 ºC and/or 1000 µatm pCO_2 increase were not sufficient to activate CAT in any tissue of non-contaminated fish, while an inhibition of this enzyme was observed in contaminated fish muscle, thus indicating that changes in CAT activity in these treatments were mostly induced by DCF, rather than by temperature and pCO_2 levels. As argued in an earlier work on *Mytilus galloprovincialis* exposed to ibuprofen (Gonzalez-Rey and Bebianno; 2001), CAT activity

inhibition in DCF exposed fish muscle could be due to an accumulation of arachidonic acid in cells due to the blockage of cyclooxygenase enzymes' activity (which are responsible for the conversion of arachidonic acid into prostaglandin), consequently leading to an excessive production of H_2O_2. Second, warming and acidification increased SOD inhibition in non-contaminated fish muscle (compared to contaminated samples) most likely due to ROS-mediated denaturation that inactivated this enzyme (Ferreira et al., 2015), but such effect was attenuated by DCF co-exposure. Third, acidification seemed to have compromised GST activity in muscle and liver, and this impairment could be due to a decreased ability to form reduced glutathione in these tissues, thus, resulting in less substrate amount for GST to act on (Gonzalez-Rey and Bebianno; 2011). Despite the impairment of the antioxidant machinery under the stress conditions mentioned above, fish muscle was the only tissue revealing increased formation of lipid peroxides (i.e. increased MDA concentration), namely, under warming or in co-exposure with acidification and DCF, thus suggesting that the enhancement of the antioxidant defences only prevented oxidative stress to some extent, leading to the consequent cell damage.

4.3.2. Chaperoning and protein degradation

Corroborating some of the present findings (i.e. muscle: acidification and warming with DCF co-exposure; liver: warming with or without co-exposure with acidification; and brain: acidification and warming, with and without DCF co-exposure), the synthesis of molecular chaperones, such as heat shock proteins, can be induced in order to repair, refold, and/or eliminate damaged proteins, thus, preventing the cellular damage promoted by the exposure to both abiotic stressors (e.g. Pimentel et al., 2015; Madeira et al. 2016a,b) and chemical contaminants (e.g. Maulvault et al., 2017), including NSAIDs (Gravel, and Vijayan, 2007). On the other hand, as observed in fish muscle under acidification alone or in the brain of fish exposed to DCF alone or combined with the other two stressors, a down-regulation can also occur under severe and/or chronic stress conditions, due to the exhaustion of the cytoprotective systems and metabolic depression (Madeira et al. 2016a), with the lack of HSPs synthesis against proteotoxic stressors being associated with cell death (Hightower, 1991). Thus, the fact that HSP70/HSC70 synthesis was inhibited in the brain when DCF acted alone suggests that warming and acidification may have reversed the effects of DCF, but not when both stressors were combined. Previous studies have highlighted that the activation of chaperones is not a straightforward process, since the threshold for induction/repression can be influenced by several factors, including stress levels, interactions with chemical contaminants, species, tissue and animal hormone levels (e.g. Mahmood et al., 2014; Madeira et al., 2016b).

Similar to HSPs, Ub synthesis can be induced to signal denatured proteins to be degraded by the proteasome, but can also be inhibited under extreme or long-lasting conditions due to

physiological collapse, as protein synthesis is an energy demanding cellular process, accounting for more than 50% of fish total oxygen consumption (e.g. Gravel, and Vijayan, 2007; Madeira et al., 2016a). Hence, such energetic expenditure may justify the decrease of Ub content in fish (liver and muscle) under the co-exposure of DCF, acidification and/or warming, as opposed to non-contaminated treatments simulating climate change effects or to DCF-exposure alone. Moreover, such impaired Ub synthesis linked with the lower cell viability and increased ENAs, further reinforces the genotoxic potential of DCF, particularly when combined with abiotic stressors.

4.3.3. Neurotoxicity and endocrine disruption

In the brain, acetylcholinesterase (AChE) can be a target for chemical contaminants, leading to the inhibition of its activity and, therefore, to failed synaptic transmission and muscle overstimulation (Schmidel et al., 2014; Topal et al., 2017). Furthermore, the exposure to abiotic stressors, such as increased temperature and pCO_2 levels, can alter AChE activity (Rosa et al., 2016). Contrasting these findings, in the current study, warming and acidification (alone or combined) did not affect AChE brain activity. This could be related to wide thresholds of physiological tolerance to temperature and pCO_2 changes of *D. labrax*, and/or to the longer acclimation and trial durations that could enable fish to cope with these changes. On the other hand, in agreement with the findings of Gonzalez-Rey and Bebianno (2014) for *M. galloprovencialis* and of Mathias et al. (2018) for *Rhamdia quelen*, DCF exposure alone induced AChE activity. Yet, this induction was counteracted by acidification and warming (i.e. no effect in DCF+Acid and DCF+Acid+Warm treatments, as well as, a significant inhibition in DCF+Warm treatment). Moreover, the changes elicited by DCF can reflect AChE activity modulation by the antioxidant machinery and VTG-like protein levels (i.e. estrogenic activity) in fish, rather than reflecting an impaired neurotransmission function (Gonzalez-Rey and Bebianno, 2014; Oliveira et al., 2015).

Although VTG is usually undetectable in male and immature individuals, its production can be elicited by the exposure to estrogenic compounds (e.g. Matozzo et al., 2008; Gonzalez-Rey and Bebianno, 2014). In agreement with previous findings, VTG synthesis was induced by DCF exposure alone or combined with warming, thus, evidencing the estrogenic potential of this contaminant (e.g. Gonzalez-Rey and Bebianno, 2014; Gröner et al., 2017). As for the effect of abiotic stressors, the enhanced VTG synthesis at warmer temperatures in both non-contaminated and DCF exposed fish is consistent with previous studies involving fish species co-exposed to endocrine disrupting compounds and increased temperatures (Chandra et al., 2012; Shappell et al., 2018), and likely related to enhanced fish metabolic rates and enzymatic activities. Interestingly, acidification seemed to have had the opposite effect, inhibiting VTG synthesis when acting alone, or not significantly affecting it when co-exposed with warming and/or DCF.

Previous studies with freshwater fish species pointed out that low seawater pH levels may have a preponderant role in fish endocrine regulation (e.g. modulating the synthesis of cortisol levels and thyroid hormones which are, in turn, linked to the VTG induction; Kwong et al., 2014; McCormick and Bradshaw, 2006). Yet, little is still known regarding the interactive effects of acidification and estrogenic contaminants co-exposure, thus, calling for the need to further investigate the effects of environmental stressors on fish endocrine system and reproduction.

4.4. Integrating the different ecotoxicological responses

As evidenced in the present study, drawing general conclusions based on different biomarker responses is not evident since: i) some biomarkers interact with each other in complex biological mechanisms; ii) biomarkers can respond differently in each tissue according to their baseline levels; iii) an environmental stressor can elicit distinct responses when acting alone or in combination with other environmental stressor; and iv) a given stressor can activate biomarker responses to a certain extent, but also inactivate them when the degree of severity/chronicity exceeds the ability of an organism to cope with that stressor, thus either up or down biomarker regulations can indicate a lower animal fitness (Serafim et al., 2012; Ferreira et al., 2015; Madeira et al., 2016a). In this way, integrating the responses of different biomarkers (IBRs) can be a useful tool, highlighting patterns that are not evident when analysing individual biomarker responses and providing a general insight of the way organisms are dealing with the exposure to environmental stressors (Serafim et al., 2012; Ferreira et al., 2015; Madeira et al., 2016a).

The fact that in the current study, the muscle (as well as the whole organisms) revealed lower mean IBR than liver and brain, is not in agreement with the trend reported by Madeira et al. (2016a), as higher IBR responsiveness was generally associated to aerobic tissues, such as muscle, gills and liver. Yet, the higher responsiveness of liver and brain observed in the present study may also be partially related to the number of biomarkers that were used to compute the IBR in each tissue (i.e. six biomarkers were used to compute the IBR in whole organism and muscle, while seven biomarkers were used for liver and brain), as the total IBR index value corresponds to the sum of all areas that connect to consecutive biomarker scores (Guerlet et al., 2010). Indeed, although previous literature does not propose an appropriate number of biomarkers to be used in IBR computations, Serafim et al. (2012), pointed out that the "relative weight" of each biomarker response is influenced by the number of biomarkers included in IBR calculations, therefore, largely affecting the final IBR value, and with a higher number of biomarkers generally yielding higher IBR indexes.

Regardless of the number/type of analysed biomarkers, overall, higher IBR values have been associated to the exposure to both chemical contaminants (Serafim et al., 2012; Ferreira et al., 2015; Ács et al., 2016) and increased temperatures (Kamel et al., 2014; Madeira et al., 2016a),

whereas no studies with biomarkers' IBR linked to acidification were found. Indeed, DCF dietary exposure was, in overall, associated to higher IBR values for the whole organism, muscle and brain, indicating a decreased fish health condition, thus, confirming the toxicological attributes of this contaminant. On the other hand, warming alone resulted in higher IBR value in the liver, and that is likely related to increased metabolism and intense enzymatic activity induced by increased temperatures in this organ.

Results pointed out that the selected biomarkers for the whole organism (i.e. K, HSI, BB_{ratio} and haematological parameters) were more responsive to the co-exposure of DCF and climate change abiotic stressors. In contrast, less consistent tissue-specific patterns were found in muscle, liver and brain, especially when DCF co-exposure occurred due to the high variability of molecular responses observed (i.e. up- or down-regulations). The overall higher IBR values registered in treatments simulating warming alone (in liver and brain) or in co-exposure with DCF (in liver and muscle) evidenced fish inability to compensate the stress induced by a 5 ºC increase. On the other hand, the role of acidification in lowering fish fitness/physiological condition was not so evident. The lower IBR values found in fish brain exposed to DCF+Acid and DCF+Warm treatments suggested that, despite the elicited biomarker changes, fish were able to better cope with stressors than those exposed to DCF, acidification and warming acting alone or to the three stressors simultaneously, likely due to the activation of antioxidant/detoxifying (GST) and chaperoning (HSP70/HSC70) mechanisms. Although the effects of warming on fish muscle, at a first glance, may seem to have been reversed by the co-exposure to acidification and DCF (i.e. lower IBR index value of DCF+Acid+Warm treatment), the IBR reduction can be explained by the inhibition of CAT activity and Ub synthesis in relation to the Control treatment and, therefore, may not be necessarily indicative of a fish better health condition. The same argument can also be applied to the lower liver IBR under acidification (i.e. in Acid and DCF+Acid treatments), as the first treatment triggered the inhibition of SOD and GST activities, as well as VTG content, whereas the second treatment inactivated SOD activity and Ub synthesis. Such findings highlight that, despite providing a broader overview of fish physiological condition, the use of IBRs has also limitations, as it provides a qualitative (and not quantitative) evaluation of the stress degree induced in fish, therefore, requiring critical interpretation and background knowledge (Serafim et al., 2012).

5. Conclusions

This study provided novel data and a contribution to fill a major research gap of the present: the interactive effects between climate change and chemical contaminants on the ecotoxicological responses of marine fish species. Results confirmed that the juvenile marine fish responses (i.e. animal fitness, immunity, cellular defence and scavenging mechanisms) to DCF dietary exposure

are strongly affected by increased temperature (+5 ºC) and pCO_2 (+1000 µatm). Such alterations can correspond to either the enhancement of biomarker responses (e.g. erythrocytes viability further reduced, as well as, GST brain activity and Ub muscle synthesis further increased by the combination of DCF and acidification and/or warming) or to an inhibition (e.g. lower CAT and SOD inhibition when DCF was combined with acidification and/or warming, as well as lower VTG synthesis when DCF was combined with warming, compared to DCF acting alone). Based on the integrated approach used in this study (i.e. the IBRs), which combined the different biomarker responses in an organism or tissue, overall, DCF exposure induced more severe stress responses when co-exposed with warming, compared to DCF acting alone or combined with acidification. Despite IBRs proved to be a helpful tool to better understand the severity of the three studied stressors (revealing trends that were not so clear when analysing biomarker responses individually), the limitations of their use were also evidenced in this study.

Finally, to sum up, the distinct effects promoted by DCF exposure, acidification and warming acting alone or combined highlighted the need to consider interactions between environmental stressors in future ecotoxicological studies. Such knowledge will provide wider insights on the toxicological impacts of climate change, as environmental stressors will unlikely occur in isolation, nor will the alterations of environmental conditions affect marine ecosystems in the same way across the planet.

Ethical statement

Fish trials were conducted according to legal regulations (EU Directive 2010/63), and approved by the Ethical Committee of the Faculty of Sciences of the Lisbon University, overseen by the Portuguese National Competent Authority (Direção-Geral de Alimentação e Veterinária, DGAV). All researchers and technicians involved in the maintenance, handling and sampling of live animals were certified in Laboratory Animal Sciences, by the Federation of European Laboratory Animal Science Associations (FELASA).

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Part 2.

Bioaccumulation of diclofenac in juvenile seabass (*Dicentrarchus labrax*) under warming and acidification

A special note regarding DCF's bioaccumulation and elimination

During the trial presented in **Chapter 3. Part 1.**, fish tissue samples were also collected from each treatment (at days 0, 7, 14 and 28 of DCF dietary exposure, as well as at day 35 corresponding to the last day of a 7 day clearance period, during which fish were fed with a non-contaminated diet, i.e. CTR feed), in order to determine DCF bioaccumulation and elimination. DCF concentration in fish tissues was determined through high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS), according to the methodology previously optimized and described in detail by Cunha et al. (2017). Despite results evidenced acceptable quality control parameters, confirming the accuracy (i.e. average recovery percentage of 83%) and inter-day repeatability (i.e. relative standard deviations, %RSD, between 11% and 18%) of the analytical methodology used, fish tissues from all treatments did not reveal positive values for DCF (i.e. values were either bellow detection, with LOD = 0.1 ng kg^{-1}, or bellow quantification, with LOQ = 0.3 ng kg^{-1}). Such absence of positive results did not allow the preparation of a manuscript entirely devoted to DCF's bioaccumulation and elimination in marine fish species. Yet, it was decided that this matter still deserved to be discussed throughout this PhD thesis, in order to provide potential explanations for such unexpected results, as well as solutions to adequately estimate DCF's toxicokinetics in the future (see also **Chapter 8. Future Perspectives**).

Based on DCF's chemical properties (i.e. relatively lipophilic, with estimated pKa = 4.1 and log KoW = 4.5; PubChem, 2018), as well as the available literature, reporting bioconcentration factors (BCF) between 4 and 10 for DCF (e.g. Schwaiger et al., 2004; Cuklev et al., 2012; Memmert et al., 2013; Peake et al., 2016), it was expected to observe a cumulative increase of DCF (even if to a low extent) in fish tissues over time. However, there are some potential explanations for this lack of agreement between the results obtained in this PhD thesis and those found in literature. First, pharmaceutical active drugs have been assumed to be mostly uptaken by marine biota through water and, thus, the majority (if not all) of studies performed so far on DCF's bioaccumulation only focused on water exposure. Hence, in addition to the previously reported dose-dependency effect (e.g. Schwaiger et al., 2004; Memmert et al., 2013), DCF bioaccumulation in fish may also vary according to the exposure route, with dietary exposure (i.e. uptake via ingestion) resulting in much lower tissue burdens compared to water exposure (i.e. uptake via inhalation). As it is later described in **Chapter 4.**, this trend was indeed verified in the trial focused on the antidepressant venlafaxine (VFX) and during which both water and dietary exposures were simulated (i.e. higher VFX tissue burdens following water exposure; Maulvault et al., 2018). Hence, this hypothesis calls for the need to further explore the differential bioaccumulation and ecotoxicological responses of pharmaceutical active drugs following distinct compound exposure pathways. Second, and somewhat in line with the previous point, by being an acidic compound, DCF in the form of sodium salt (the one used in the preparation of

contaminated fish feeds; see **Chapter 3. Part 1.**) is rapidly dissolved in the stomach of mammals (including humans), but immediately precipitates due to the low pH of the gastric fluid, therefore, strongly affecting the dissolution and subsequent absorption of DCF parental compound at the duodenum (Altman et al., 2015; Kataoka et al., 2016). Hence, even though most studies on the absorption of pharmaceuticals have been conducted on mammalian subjects, the physiological and endocrinological similarities between humans and fish suggest that the same principle may also apply to the last. Thus, it may be hypothesized that the absence of DCF tissue bioaccumulation in fish tissues could be related to the fact that this compound was administered via feed (as opposed to most studies on marine biota exposed to DCF), as well as to the molecular form of DCF used in this study (i.e. DCF-sodium salt).

Once pharmaceuticals are ready for absorption at the duodenum, the subsequent fate of these compounds is largely depend on the mechanisms that an organism disposes to metabolize and/or excrete them. As such, another explanation for the present results and, perhaps, the most plausible one, is the fact that DCF in its parental form (and regardless of the salt form it is presented) has a very short half-life (~2 hours) as it is readily metabolized in the liver, engaging in phase I and phase II biotransformation processes and, later, being mostly excreted in the form of different DCF metabolites (i.e. 6 described in humans: 4′-hydroxy-DCF, 5′-hydroxy-DCF, 3′-hydroxy-DCF, 4′,5-dihydroxy-DCF, 3′-OH-4′-OCH-DCF and 4′-OH-3′-OCH_3-DCF) and conjugates (glucuronide and sulfate conjugates; Lee et al., 2012; Vieno and Sillanpää, 2014; Peake et al., 2016). In this way, the negative results obtained in this study (that only determined fish tissue concentrations of DCF in its parental/unchanged form) may be attributed to the rapid metabolization and subsequent excretion of this compound via urine and/or faeces. Indeed, in a study performed with rainbow trout (*Oncorhynchus mykiss*) exposed to DCF (for 10 days, via intraperitoneal injection), apart from DCF in its parental form, two hydroxylated DCFmetabolites (4'-hydroxydiclofenac and 4;5-hydroxydiclofenac) and 6 DCF-conjugate products were also identified in fish bile, with acyl glucuronide conjugates being the predominant forms of this compound in the study (Kallio et al., 2010). Unfortunately, in our study it was not possible to quantify the presence of DCF metabolites and conjugates. Hence, this highlights the importance of further unveiling the detoxification mechanisms of pharmaceuticals (and other xenobiotics) in fish species, particularly focusing on the determination of metabolites that can often be more persistent and toxic than the parental compound itself.

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CHAPTER 4.

ANTIDEPRESSANT DRUGS: VENLAFAXINE AS A CASE STUDY

Part 1.

Antidepressants in a changing ocean: Venlafaxine uptake and elimination in juvenile fish (*Argyrosomus regius*) exposed to warming and acidification conditions

Manuscript 4.

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Abstract

The presence of antidepressants, such as venlafaxine (VFX), in marine ecosystems is increasing, thus, potentially posing ecological and human health risks. The inherent mechanisms of VFX uptake and elimination still require further understanding, particularly accounting for the impact of climate change-related stressors, such as warming and acidification. Hence, the present work aimed to investigate, for the first time, the effects of increased seawater temperature ($\Delta T^{o}C$ = +5 ºC) and pCO_2 levels (ΔpCO_2 ~1000 µatm, equivalent to ΔpH = -0.4 units) on the uptake and elimination of VFX in biological tissues (muscle, liver, brain) and plasma of juvenile meagre (*Argyrosomus regius*) exposed to VFX through two different exposure pathways (via water, i.e. [VFX] ~20 µg L^{-1}, and via feed, i.e. [VFX] ~160 µg kg^{-1} dry weight, dw). Overall, results showed that VFX can be uptaken by fish through both water and diet. Fish liver exhibited the highest VFX concentration (126.7 ± 86.5 µg kg^{-1} and 6786.4 ± 1176.7 µg kg^{-1} via feed and water exposures, respectively), as well as the highest tissue:plasma concentration ratio, followed in this order by brain and muscle, regardless of exposure route. Both warming and acidification decreased VFX uptake in liver, although VFX uptake in brain was favoured under warming conditions. Conversely, VFX elimination in liver was impaired by both stressors, particularly when acting simultaneously. The distinct patterns of VFX uptake and elimination observed in the different scenarios calls for a better understanding of the effects of exposure route and abiotic conditions on emerging contaminants' toxicokinetics.

Keywords: emerging contaminants, antidepressant, venlafaxine, bioaccumulation, climate change, fish.

1. Introduction

Over the last years, pharmaceuticals and personal care products (PPCPs) have been widely detected in marine ecosystems (e.g. Arpin-Pont et al., 2016; Rodriguez-Mozaz et al., 2017). The frequent detection of PPCPs in the marine environment can be attributed to two main reasons: i) increased human usage of PPCPs along with increased world population; and ii) these chemical contaminants are not completely eliminated at conventional wastewater treatments plants (WWTPs) (Gros et al., 2012) despite the constant improvements of technology. Indeed, the cost associated to removal of contaminants in WWTPs is so high that cities municipalities cannot afford to implement such novelties, and therefore PPCPs and other chemicals are still barely removed reaching water bodies. As emerging contaminants, PPCPs lack regular environmental monitoring data and their presence in seafood species is not regulated yet. Among PPCPs, the antidepressant venlafaxine (VFX), a serotonin-norepinephrine selective reuptake inhibitor (SNRI), is one of the most commonly prescribed psychiatric drugs used to treat depression and anxiety disorders in humans, being frequently detected in the aquatic environment (Álvarez-Muñoz et al., 2015; Arpin-Pont et al., 2016; Rodriguez-Mozaz et al., 2017). Recent studies have provided compelling evidence that antidepressants can accumulate in marine biota (e.g. Álvarez-Muñoz et al., 2015), and promote adverse effects at the behavioural and physiological levels as well (e.g. Bisesi Jr. et al., 2014; Best et al., 2014; Bidel et al., 2016). Most of the studies about uptake and elimination mechanisms of these contaminants have focused on their exposure via water (e.g. Boillot et al., 2015; Huerta et al., 2016; Valdés et al., 2016; Serra-Compte et al., 2018), thus not considering contaminant bioaccumulation through trophic transfer (i.e. dietary exposure; e.g. Heynen et al., 2016; Boström et al., 2017). Yet, the later also represents a major pathway of contaminant exposure, particularly in predatory fish species (Brooks, 2014). Furthermore, the potential interactive effects between these contaminants and other environmental stressors are still unclear, although they can play a key role on chemical contaminants' availability and toxicity to biota (e.g. Noyes et al. 2009; Marques et al., 2010; Maulvault et al., 2016). Understanding how marine species will cope with the presence of multiple environmental stressors represents one of the main ecological concerns and research challenges, since climate change-related effects, such as seawater warming and acidification, are already evident in many regions of the world, posing a great threat to marine life (IPCC, 2014). Such changes are expected to worsen in the coming 50-100 years, for instance, increasing seawater temperature as high as 5 ºC, as well as CO_2 partial pressures (pCO_2) up to 1000 µatm which leads to a seawater pH drop, a process known as ocean acidification (IPCC, 2014; McNeil and Sasse, 2016). Depending on the region, each stressor can occur alone or in combination with other ones, representing additional challenges to the resilience of marine ecosystems.

Seawater warming and acidification can alter contaminants' bioavailability in sediments and in water as well as the marine specie's physiological status, which may affect the way that marine organisms cope with the presence of chemical contaminants like compound uptake, retention and detoxification rates (e.g. Marques et al., 2010; Maulvault et al., 2016, 2017; Sampaio et al., 2016). Such alterations were clearly evidenced in a recent study on European seabass (*Dicentrarchus labrax*; e.g. Maulvault et al., 2016), which revealed enhanced bioaccumulation and impaired elimination of MeHg under warmer temperatures, as well as increased risks to seafood consumers. Therefore, it is of paramount importance to gather relevant data from environmental pollutants, particularly from those less well studied, such as PPCPs, enabling a better prediction of the potential implications of climate change at the ecotoxicological and public health levels.

In this context, the aim of this study was to assess, for the first time, the effects of increased seawater temperature ($\Delta T^{o}C$ = +5 ºC) and pCO_2 levels (~1000 µatm, equivalent to ΔpH = -0.4 units) on VFX uptake and elimination mechanisms in fish tissues (muscle, liver and brain) and plasma of juvenile meagre (*Argyrosomus regius*) exposed to the contaminant through two different exposure pathways (via water, i.e. [VFX] ~20 µg L^{-1}, and via feed, i.e. [VFX] ~160 µg kg^{-1} dry weight, dw). The selection of juvenile *A. regius* as model species was based on the following criteria: i) it is a predatory fish species, inhabiting temperate estuaries and coastal areas, thus, likely accumulating high levels of chemical contaminants (FAO, 2017); ii) environmental variations and pollution are known to be particularly deleterious to species' early life stages (including larvae and juveniles), compromising their recruitment and ecological success; and iii) it is a commercially important species and, therefore, the data obtained can be linked to seafood safety.

2. Materials and Methods

2.1. Feeds (CTR and VFX-enriched) and VFX stock solutions

Two feed batches with the same nutritional composition were manufactured by the company SPAROS Lda (Olhão, Portugal): a non-contaminated feed (CTR feed) and a VFX-enriched feed. Both feeds were elaborated in order to mimic a commercial fishmeal-rich formulation for juvenile marine fish (48% crude protein and 18% crude fat; detailed feed composition is presented in **Annex 5, Table A.5.1.**). Briefly, all powder ingredients were grinded (<200 micron) in a micropulverizer hammer mill (Hosokawa Micron, SH1, The Netherlands), and mixed with fish oil in a paddle mixer (Mainca RM90, Spain). The feed mixture was further humidified with 25% deionized water at room temperature, and then extruded at 2.0 mm by means of a low-shear extruder (P55, Italplast, Italy). After extrusion, feed pellets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). Then, a batch of CTR feed was subsequently enriched with a solution of venlafaxine hydrochloride ($C_{17}H_{27}NO_2 \cdot HCl$, >98%, Sigma-Aldrich)

dissolved in ethanol, and further diluted with deionized water (total volume of 100 mL). A VFX nominal concentration of approximately 160 µg kg^{-1} on a dry weight basis (dw) was selected for the VFX-enriched feed (i.e. corresponding to a concentration ~4 times higher than the VFX levels found in species inhabiting contaminated coastal areas, susceptible to accumulate this contaminant, and that are natural preys of juvenile meagre, to assure that VFX bioaccumulation to detectable levels was elicited during the timeline of the trials; Martínez Bueno et al., 2014; Álvarez-Muñoz et al., 2015). Feed enrichment was performed by top-coating the pellets with the VFX solution using a pressurized spraying container (standard flat-fan nozzle; size 250 micron; pressure 6 bar). VFX concentration was determined in VFX-enriched feed, as well as in CTR feed to assure that no external contamination occurred during the preparation of CTR feed (**Table 4.1.1.**).

For treatments simulating VFX exposure via water, a stock solution of VFX was prepared to perform the daily seawater spiking during the 28 days of exposure, by dissolving venlafaxine hydrochloride ($C_{17}H_{27}NO_2 \cdot HCl$, >98%, Sigma-Aldrich) with deionized water (total volume of 500 mL), in order to achieve a nominal VFX concentration of 20 µg L^{-1} in each incubating tank. Despite this value is above the concentrations usually found in seawater, the selection of VFX nominal concentration was mostly based on the order of magnitude of the lowest VFX concentration previously reported to cause significant behavioural effects in fish following short-term VFX exposure (50 µg L^{-1}; Bisesi Jr et al., 2014). The total volume of VFX solution used to perform the daily spiking was adjusted considering that the steady state of this compound was reached after 24h, and accounted for the possible compound losses due to the daily 25% seawater renewal in each tank.

2.2. Fish rearing and acclimation

Argyrosomus regius specimens were reared until juvenile stage, at the aquaculture pilot station of the Portuguese Institute for the Sea and Atmosphere (EPPO-IPMA, Olhão, Portugal) using routine hatchery conditions. Subsequently, fish with similar biometric characteristics (total length: 6.8 ± 0.4 cm; total weight 2.6 ± 0.5 g) were transported to the aquaculture facilities of Laboratório Maritimo da Guia (MARE-FCUL, Cascais, Portugal), where they were randomly and equitably distributed in 21 rectangular shaped incubating glass tanks (50 L each, total volume). The incubating tanks were organized in groups of three tanks, which corresponded to one treatment (i.e. 3 replicates x 7 treatments = 21 tanks in total; **Figure 4.1.1.**; see the description of each treatment in **section 2.3.**).

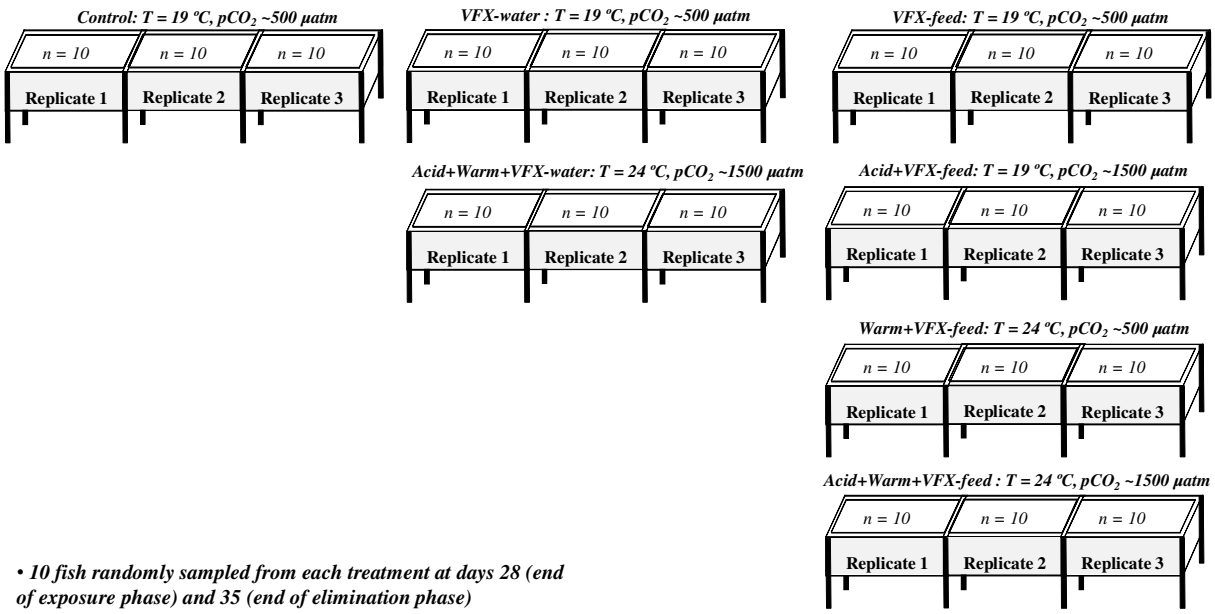

Figure 4.1.1. Experimental setup. Abbreviations: Acid – simulated acidification (i.e. pCO_2 ~1500 µatm, equivalent to pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC); VFX-water – fish exposed to venlafaxine via water; VFX-feed – fish exposed to venlafaxine via feed.

Animal density was kept below 5 g body weight L^{-1} in each tank in order to avoid physiological stress related to high animal density. To maintain seawater quality, each incubating tank had independent functioning, being equipped with a protein skimmer (Reef SkimPro, TMC Iberia, Portugal), UV disinfection (Vecton 300, TMC Iberia, Portugal), biological filtration (model FSBF 1500, TMC Iberia, Portugal) and chemical filtration (activated carbon, Fernando Ribeiro Lda, Portugal; except in treatments simulating VFX exposure via water; i.e. VFX-water). Dead fish and faeces were daily removed, and seawater was partially renewed (25% of the total tank volume). Ammonia, nitrite and nitrate levels were daily checked using colorimetric tests (Tropic Marin, USA), and kept below detectable levels, with the exception of nitrates, which were kept below 2.0 mg L^{-1}. Seawater total alkalinity was also measured in each tank on a weekly basis, following the protocol previously described (Sarazin et al., 1999), and the combination of total alkalinity (AT) and pH was used to calculate carbonate system parameters (average values obtained for each treatment can be consulted in **Annex 5, Table A.5.2.**). Each tank had independent temperature and pH control, and daily adjustments of these parameters were performed by means of: i) temperature - an automatic seawater refrigeration system (± 0.1 °C; Frimar, Fernando Ribeiro Lda, Portugal), as well as submerged digital thermostats (200W, V2Therm, TMCIberia, Portugal); ii) pH - individual pH probes (GHL, Germany) connected to a computerized pH control system (± 0.1 pH units; Profilux 3.1N, GHL, Germany), which monitored seawater pH in each tank every 2 s and adjusted whenever needed, via submerged air stones, by injecting CO_2 (Air Liquide, Portugal; to decrease pH) or CO_2-filtered aeration (to increase pH) using air pumps (Stella 200, Aqua One Pro, Aqua Pacific UK Ltd, United Kingdom).

Prior to trials, fish were acclimated to laboratory conditions for a period of 30 days, being fed with CTR feed (2% of average body weight, bw) and kept under the following abiotic conditions: i) dissolved oxygen (DO) > 5 mg L^{-1}; ii) temperature (T ºC) = 19.0 ± 0.5 °C; iii) pH = 8.00 ± 0.10; iv) salinity = 35 ± 1 ‰; and v) photoperiod = 12L:12D (12 hours light:12 hours dark). Temperature, pH, salinity and DO were daily checked using a multi-parameter measuring instrument (Multi 3420 SET G, WTW, Germany).

2.3. VFX exposure and elimination

The effect of warming and acidification acting together (i.e. the worst-case scenario) was assessed in the treatments simulating both VFX exposure routes (i.e. seawater and dietary exposures; Acid+Warm+VFX-water and Acid+Warm+VFX-feed). Yet, due to experimental limitations, only the exposure pathway through feed was selected to investigate the single effects of temperature and pH (i.e. Acid+VFX-feed and Warm+VFX-feed). The exposure via VFX-enriched feed was selected for this purpose, because: a) there is currently a lack of data on contaminant bioaccumulation through trophic transfer (i.e. dietary sources), as most of the studies focus on contaminant exposure through inhalation (i.e. water exposure); b) diet can be an important source of contaminant exposure, particularly in predatory species, often leading to more notorious toxicological effects than those promoted by contaminant exposure through inhalation (e.g. Arnot and Gobas, 2004; Brooks, 2014).

One week before initiating the trial, seawater temperature and pH were slowly adjusted (+1 ºC and -0.1 unit per day), until reaching 24 ºC and ~1500 µatm pCO_2 (equivalent to pH = 7.6 units) in tanks simulating climate change conditions (i.e. treatments Acid+VFX-feed, Warm+VFX-feed and Acid+Warm+VFX-feed and Acid+Warm+VFX-water; **Figure 4.1.1.**), according to the projections of the Intergovernmental Panel for Climate Change (scenario RCP8.5; IPCC, 2014), as well as considering the intervals of future CO_2 amplification scenarios described by McNeil and Sasse (2016).

Seven treatments were carried out (n = 10 animals per replicate tank; **Figure 4.1.1.**), simulating the reference temperature (i.e. 19 ºC) and pCO_2 (~500 µatm; pH = 8.0 units) conditions (i.e. temperature and pCO_2 conditions normally used in juvenile meagre rearing), as well as the projected seawater warming (ΔTºC = +5 ºC) and acidification (ΔpCO_2 ~1000 µatm; equivalent to ΔpH = -0.4 units): i) Control treatment, i.e. fish daily fed with CTR feed, and simulation of the reference temperature and pH conditions; ii) VFX-water treatment, i.e. fish daily fed with CTR, seawater daily spiked with a VFX stock solution, to achieve a VFX final concentration of 20 µg L^{-1}, and simulation of the reference temperature and pH conditions; iii) Acid+Warm+VFX-water treatment, i.e. fish daily fed with CTR, seawater daily spiked with a VFX stock solution (nominal concentration = 20 µg L^{-1}), and simulation of warming and acidification (24ºC and ~1500 µatm

pCO_2); iv) VFX-feed treatment, i.e. fish daily fed with VFX-enriched feed, and simulation of the reference temperature and pH conditions; v) Acid+VFX-feed treatment, i.e. fish daily fed with VFX-enriched feed, and simulation of acidification (1500 µatm pCO_2); vi) Warm+VFX-feed treatment, i.e. fish daily fed with VFX-enriched feed, and simulation of warming (24 ºC); vii) Acid+Warm+VFX-feed treatment, i.e. fish daily fed with VFX-enriched feed, and simulation of warming and acidification (24ºC and ~1500 µatm pCO_2). After 28 days of exposure to VFX (day 28), a contaminant elimination phase was also carried out for a period 7 days (until day 35), i.e. fish were fed with CTR feed in all VFX-feed and VFX-water treatments, and VFX seawater spiking was stopped in VFX-water treatments (seawater also partially renewed on a daily basis during this phase, i.e. 25% of the total tank volume). During the exposure and elimination phases, fish were daily fed with the same amount (2% bw) of the corresponding feed, and feed consumption was regularly monitored throughout the entire experiment, to assure that all feed was consumed by fish from every treatment (no reduction of fish consumption was observed). Seawater abiotic parameters were daily monitored and adjusted to adequate levels whenever needed, as described above. No mortality was observed during the 35 days of the trial.

Ten fish were randomly sampled from each treatment (out of the 3 replicate tanks constituting one treatment) at days 28 and 35, and euthanized by immersion in an overdosed MS222 solution (2000 mg L^{-1}; Sigma-Aldrich, USA) buffered with sodium bicarbonate (1 g of $NaHCO_3$ and 1 g of MS222 per litre of seawater) for 10 min. Euthanized fish were measured (average weight, W, and total length, TL, are shown in **Table 4.1.2.**), and blood was collected by puncture of the caudal vein and centrifuged (4 ºC, 15 min, 10,000 *g*).

Plasma samples were then collected, pooled in two composite samples (plasma of 5 individuals per pool, $n = 2$) and kept at -80 ºC until further analyses. Fish were subsequently dissected to collect the muscle, liver and brain tissues, and two composite samples were also performed for each of these three tissues (i.e. muscle, liver or brain of 5 individuals per pool, $n = 2$). Composite samples were then freeze-dried at −50 °C, 10^{-1} atm of vacuum pressure for 48 h (Power Dry LL3000, Heto, Czech Republic), homogenized and stored at -80 °C until further analysis.

Seawater samples were also collected from each replicate tank (treatment) during the exposure (i.e. days 14 and 28) and elimination (i.e. day 35) phases for VFX quantification, in order to assure: i) a steady VFX concentration in tanks/treatments simulating VFX exposure via water (**Table 4.1.1.**); and ii) no external contamination (apart from dietary exposure) was taking place in tanks/treatments simulating VFX exposure via feed (**Table 4.1.1.**).

Table 4.1.1. Venlafaxine concentration (mean ± standard deviation) in feed (µg kg^{-1} dry weight) and seawater samples (µg L^{-1}) collected from each treatment, as well as in non-contaminated fish tissues (Control treatment). Abbreviations: CTR feed: non-contaminated control feed; VFX-enriched feed: venlafaxine contaminated feed; VFX-feed – fish exposed to venlafaxine via feed; VFX-water – fish exposed to venlafaxine via seawater; Acid – simulated acidification (i.e. pCO_2 ~1500 µatm, equivalent to pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC).

		VFX concentrations		
Feed (µg kg^{-1}, dw)	CTR feed		< LOD	
	VFX-enriched feed		161.7 ± 17.1	
Seawater (µg L^{-1})		Day 14	Day 28	Day 35
	Control	< LOD	< LOD	< LOD
	VFX-feed	< LOD	< LOD	< LOD
	Acid+VFX-feed	< LOD	< LOD	< LOD
	Warm+VFX-feed	< LOD	< LOD	< LOD
	Acid+Warm+VFX-feed	< LOD	< LOD	< LOD
	VFX-water	19.7 ± 1.6	19.2 ± 1.6	16.0 ± 1.4
	Acid+Warm+VFX-water	23.2 ± 4.7	22.3 ± 2.1	15.9 ± 1.4
Fish plasma (µg L^{-1}) and tissues (µg kg^{-1}, dw)	Control		Day 28	Day 35
		Plasma	< LOD	< LOD
		Brain	2.6 ± 2.1	< LOD
		Liver	21.8 ± 0.6	23.0 ± 0.6
		Muscle	< LOD	< LOD

Table 4.1.2. Biometric data (mean ± standard deviation) of fish collected from each treatment at days 28 and 35 of the trial. Different lower case letters indicate significant differences between Control and VFX-feed treatments, whereas upper cases letters indicate significant differences between Control and VFX-water treatments ($p < 0.05$). Abbreviations: W – Weight; TL – total length; VFX-water – fish exposed to VFX via seawater; VFX-feed – fish exposed to VFX via feed; Acid – simulated acidification (i.e. pCO2 ~1500 µatm, equivalent to pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC).

		W (g)	TL (cm)
Day 28	Control	$3.7 \pm 1.1^{b, B}$	$7.3 \pm 0.7^{ab, B}$
	VFX-feed	2.2 ± 0.4^{b}	6.5 ± 0.4^{b}
	VFX-feed+Acid	4.4 ± 2.8^{ab}	7.9 ± 1.4^{ab}
	VFX-feed+Warm	6.5 ± 1.2^{a}	8.9 ± 0.5^{a}
	VFX-feed+Acid+Warm	6.7 ± 2.0^{ab}	9.0 ± 1.0^{a}
	VFX-water	2.9 ± 0.7^{B}	6.8 ± 0.4^{B}
	VFX-water+Acid+Warm	7.1 ± 2.1^{A}	9.3 ± 0.9^{A}
Day 35	Control	$2.6 \pm 0.8^{b, B}$	$6.8 \pm 0.6^{c, B}$
	VFX-feed	2.4 ± 0.8^{b}	6.8 ± 0.8^{c}
	VFX-feed+Acid	2.8 ± 0.8^{b}	7.0 ± 0.6^{c}
	VFX-feed+Warm	5.0 ± 0.7^{a}	8.5 ± 0.4^{b}
	VFX-feed+Acid+Warm	7.1 ± 1.9^{a}	9.6 ± 0.6^{a}
	VFX-water	3.1 ± 1.0^{B}	7.4 ± 0.7^{B}
	VFX-water+Acid+Warm	7.6 ± 1.3^{A}	9.8 ± 0.6^{A}

2.4. Venlafaxine determination

Seawater samples were filtered by PVDF syringe filters 0.22 µm (Merck Millipore) and VFX was quantified by direct injection in UPLC-QqLIT according to the methodology described by Gros et al. (2012). VFX levels in feed (CTR and VFX-enriched) were determined following an extraction method adapted from Jakimska et al. (2013), and further quantified by UPLC-QqLIT according to Gros et al. (2012). For the determination of VFX in the fish plasma and tissues (brain, liver and muscle) at day 28 and day 35 of the experiment, the following extraction protocols were used: i) Plasma: 50 µL of plasma were mixed with 50 µL of methanol and centrifuged (5340 *g*, 10 min, 4 ºC), 60 µL of supernatant were transferred to an insert, and 0.6 µL of a 1 ng μL^{-1} VFX-d6 standard solution was added before the analysis; ii) Brain, liver and muscle: 50 mg of freeze-dried tissue (or 25 mg in the case of brain) were extracted with 1 mL methanol:water (75:25, *v/v*) in an ultrasonic bath during 15 min. Then samples were centrifuged (8000 *g*, 15 min, 4 ºC). This extraction procedure was repeated three times. All the supernatants were combined, evaporated until dryness under a gentle stream of nitrogen and reconstituted in 50 mL ultra-pure water with 0.1% formic acid. A solid phase extraction (SPE) using Oasis MCX (3 cc, 60 mg) (Waters) was performed for sample clean-up. Briefly, cartridges were conditioned with 3 mL methanol and 3 mL of 0.1% formic acid in ultra-pure water. Then, the reconstituted samples were percolated through the cartridges at a flow rate of 3 mL min^{-1}. The cartridges were washed with 5 mL 2% formic acid in ultra-pure water and dried under vacuum for 5 min. Finally, VFX was eluted with 6 mL 5% ammonia in methanol. The extracts were evaporated until dryness under a gentle stream of nitrogen and reconstituted in 1 mL methanol:water (50:50, *v/v*). Before analysis, extracts were filtered by PVDF syringe filters (0.22 µm) (Merck Millipore) and VFX-d6 standard solution was added as internal standard. The quantification of VFX in all samples was performed using the chromatographic method described by Gros et al. (2012). Detailed information on the validation parameters for the analysis of venlafaxine in feed, water, fish tissues and plasma is given in **Annex 5, Table A.5.3**.

2.5. Data analysis

Venlafaxine net accumulation rates (NAR; µg kg^{-1} day^{-1}) for each tissue and treatment were determined assuming that fish were exposed to steady conditions (i.e. continuous contaminant exposure, as well as seawater abiotic parameters) and using the following equation (Santana et al., 2017):

$$\mathrm{NAR_t} = \frac{[\mathrm{VFX_{t28}}]_{tissue}}{\mathrm{t}}$$

where, $[VFX_{t28}]_{tissue}$ is the average VFX concentration in fish tissues after 28 days of exposure. Furthermore, for VFX-water treatments, the bioconcentration factor (BCF) was also calculated after 28 days of VFX exposure using the following equation (in L kg^{-1}; Arnot and Gobas, 2006):

$$BCF = \frac{[\mathrm{VFX_{t28}}]_{tissue}}{[\mathrm{VFX_{t28}}]_{sewater}}$$

where, $[VFX_{t28}]_{seawater}$ is the average VFX concentration in seawater samples collected from treatments simulating VFX exposure via water, after 28 days of exposure. To facilitate comparisons with previously reported data, BCF were calculated on a wet weight (ww) basis, i.e. for brain, liver and muscle the average moisture content (79.7%, 63.1% and 79.5%, respectively) was used to convert VFX concentration values.

The percentage of VFX loss during the elimination phase (i.e. day 35), i.e. the elimination factor (EF; %) was calculated according to the following equation:

$$EF\ (\%) = 100 - \left(\frac{[\mathrm{VFX_{t35}}]_{tissue}}{[\mathrm{VFX_{t28}}]_{tissue}}\ x\ 100\right)$$

where, $[VFX_{t35}]_{tissue}$ is the VFX concentration in fish tissues after 7 days of elimination (i.e. day 35; Jebali et al., 2014). EF was considered to be 0 whenever $[VFX_{t35}]_{tissue}$ was higher than $[VFX_{t28}]_{tissue}$.

In what concerns statistical analysis, the ANOVA assumptions of normality and variances homogeneity were first investigated through the Kolmogorov–Smirnov and Levene tests, respectively. Data were Log-transformed or square-rooted whenever necessary to comply with these assumptions. Then, to assess the existence of significant differences between treatments in terms of VFX tissue concentrations, three-way ANOVA analyses were performed (for both exposure routes, i.e. VFX-water and VFX-feed treatments), using tissue (brain, liver and muscle) or tissue:plasma concentration ratios, sampling day (days 28 and 35) and treatment (reference temperature and pH, warming and/or acidification) as variables. As for biometric data (total weight and total length), NAR (day 28), BCF (day 28), EF (day 35) and VFX plasma, two-way ANOVA were used instead (for both exposure routes, i.e. VFX-water and VFX-feed treatments) to determine the existence of significant differences between treatments (biometric data: day and treatment used as variables; NAR, BCF and EF: tissue and treatment used as variables). Two-way ANOVA was also carried out to detect the presence of significant differences among treatments and sampling days in VFX plasma concentrations (results are presented in **Annex 5, Table A.5.4.**). Post-hoc Tukey HSD tests were subsequently carried out to identify significant

differences. Finally, Pearson correlation coefficients (*r*) between biometric data and NAR were also calculated. Statistical analyses were performed at a significance level of 0.05, using STATISTICA™ software (Version 7.0, StatSoft Inc., USA).

3. Results

3.1. VFX levels in feed, seawater and non-contaminated fish samples

VFX concentration in feed and seawater samples, as well as in non-contaminated fish throughout the 35 days of trial is shown in **Table 4.1.1.** An average VFX concentration of 161.7 ± 17.1 µg kg^{-1} was obtained in VFX-enriched feed, whereas, VFX was not detected in CTR feed (**Table 4.1.1.**). In seawater samples, detectable VFX levels were only found in VFX-water treatments (i.e. around 21 µg L^{-1} at days 14 and 28 and 16 µg L^{-1} at day 35; **Table 4.1.1.**). As for non-contaminated fish samples (i.e. Control treatment), despite VFX was not detected in seawater samples nor in CTR feed, liver and brain of control fish samples exhibited low, but still quantifiable levels of VFX (i.e. around 22 µg kg^{-1} in the liver at days 28 and 35, and around 2 µg kg^{-1} in the brain only at day 28). Thus, these values were subsequently accounted for when analysing data obtained in contaminated fish.

3.2. VFX uptake and elimination via water

VFX concentration in tissues of fish exposed via contaminated seawater, as well as the tissue:plasma concentration ratios are shown in **Figure 4.1.2.** and **Table A.3.**. VFX concentration significantly varied according to tissue, with liver being the primary organ for VFX uptake, regardless of seawater temperature and pCO_2 conditions (6786.4 ± 1176.7 µg kg^{-1} dw, equivalent to an average NAR of 241.6 µg kg^{-1} day^{-1} in VFX-water treatment, and 3692.1 ± 272.9 µg kg^{-1} dw equivalent to an average NAR of 131.1 µg kg^{-1} day^{-1} in Acid+Warm+VFX-water treatment; $p < 0.05$; **Figures 4.1.2.A** and **4.1.3.A**). Furthermore, significantly higher liver:plasma concentration ratios were observed in fish exposed to the reference temperature a pCO_2 conditions (5.3 ± 1.2 and 9.8 ± 2.5 at days 28 and 35, respectively; **Figure 4.1.2.C**). On the other hand, significantly lower VFX concentrations were found in the other fish tissues, particularly, in the muscle (423.3 ± 159.2 µg kg^{-1} dw equivalent to an average NAR of 15.1 µg kg^{-1} day^{-1}, and 329.2 ± 44.8 µg kg^{-1} dw equivalent to an average NAR of 11.8 µg kg^{-1} day^{-1}, in treatments VFX-water and Acid+Warm+VFX-water, respectively; **Figures 4.1.2.A** and **4.1.3.A**). Furthermore, significant and negative correlations were found between morphometric data and VFX NARs in these three tissues (*r* ranging between -0.86 and -0.95; $p < 0.05$; **Table 4.1.3.**).

Regarding the combined effects of abiotic variables, warming combined with acidification resulted in significantly higher W and TL compared to those observed in fish exposed to the reference temperature and pCO_2 conditions ($p < 0.05$; **Table 4.1.2.**), and such increase was

accompanied by an overall reduction of VFX concentrations and tissue:plasma concentration ratios ($p < 0.05$; **Figures 4.1.2.A,C**), as well as significantly lower NARs and BCFs in plasma, brain and liver tissues ($p < 0.05$; **Figures 4.1.3.A,B**).

During the elimination phase (day 35), overall, increased temperature and pCO_2 facilitated VFX elimination in the liver and muscle tissues (i.e. EF maximum values up to 32% and 22%, respectively; $p < 0.05$; **Figure 4.1.3.C**). Yet, such trend was not observed in fish brain, as no elimination was observed in this tissue, regardless of seawater temperature and pCO_2 conditions (**Figures 4.1.2.B,C**).

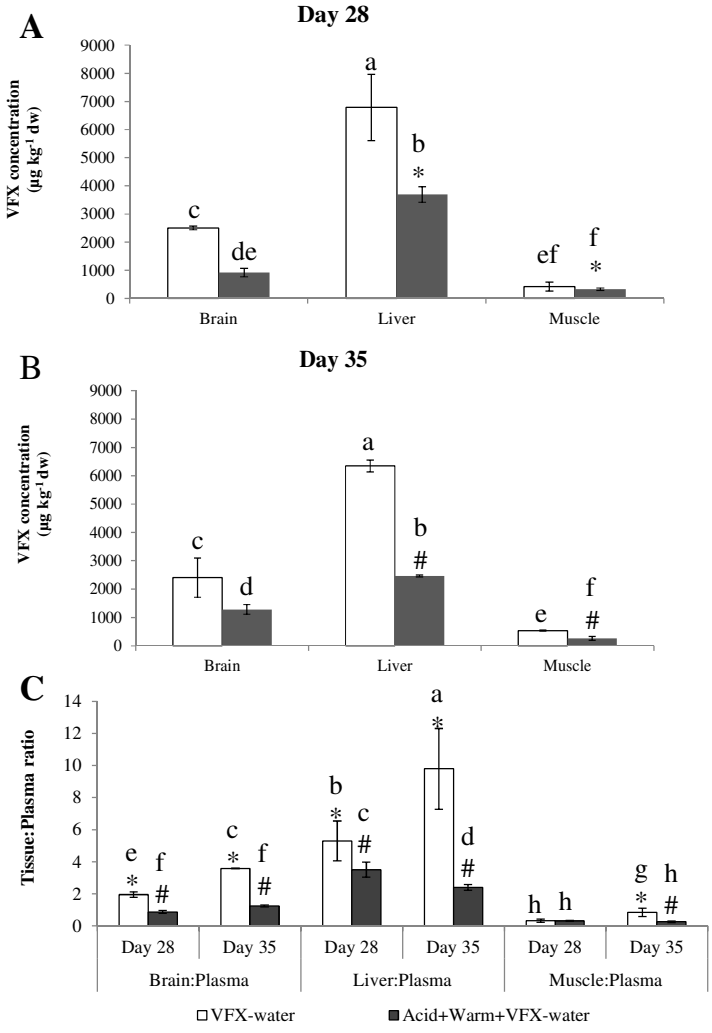

Figure 4.1.2. Venlafaxine (VFX) concentrations (µg kg^{-1} dw) in tissues of fish exposed to VFX via water at day 28 (exposure phase; A) and day 35 (elimination phase; B), as well as, tissue to plasma VFX concentration ratio at days 28 and 35 (C). Different letters indicate significant differences between tissues and treatments, whereas different symbols (* or #) indicate significant differences between day 28 and day 35 for the same tissue ($p < 0.05$). Abbreviations: Acid – simulated acidification (i.e. pCO_2 ~1500 µatm, equivalent to pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC).

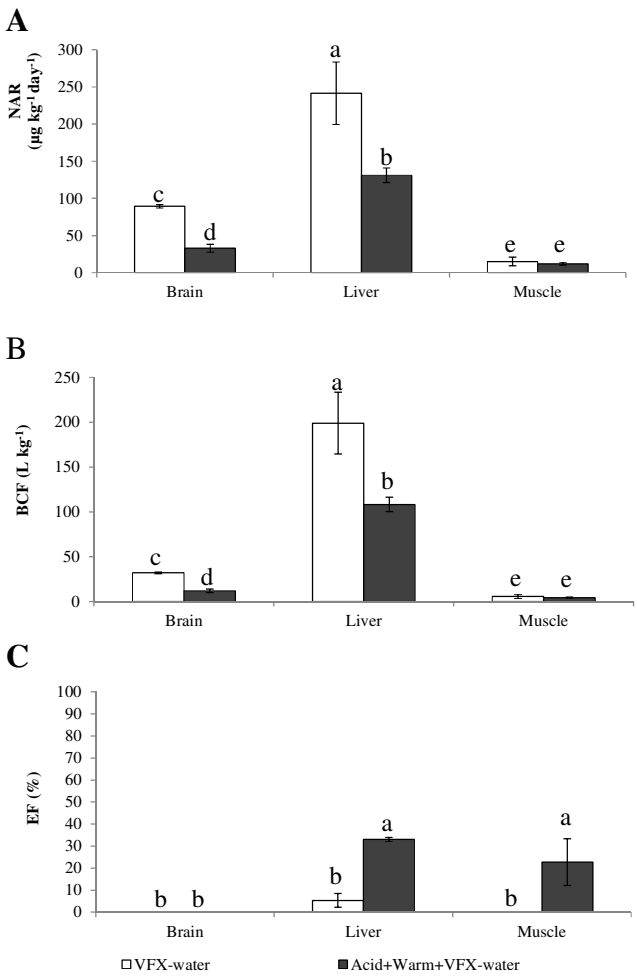

Figure 4.1.3. Venlafaxine (VFX) net accumulation rates at day 28 (NAR; µg kg^{-1} day^{-1}; A), bioconcentration factors (BCF; L kg^{-1} ww; B) and VFX elimination factors at day 35 (EF; %; C) in VFX-water treatments. Different case letters indicate significant differences ($p < 0.05$). Abbreviations: Acid – simulated acidification (i.e. pCO_2 ~1500 µatm, equivalent to pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC).

Table 4.1.3. Pearson correlation coefficients between biometric data and venlafaxine (VFX) net accumulation rate (NAR). In each column, asterisks indicate significant correlations. Abbreviations: W – weight; TL – total length; VFX-water – fish exposed to VFX via seawater; VFX-feed – fish exposed to VFX via feed.

		TW vs TL	NAR vs TW	NAR vs TL
VFX-water treatments	**Plasma**	**0.98***	**-0.86***	**-0.92***
	Brain		**-0.91***	**-0.95***
	Liver		**-0.87***	**-0.90***
	Muscle		-0.57	-0.63
VFX-feed treatments	**Plasma**	**0.97***	**-0.67***	**-0.72***
	Brain		0.31	0.27
	Liver		-0.40	-0.41
	Muscle		0.01	0.00

3.3. VFX uptake and elimination via feed

VFX concentration in tissues of fish exposed via feed, as well as the tissue:plasma concentration ratios are shown in **Figure 4.1.4.** and **Table A.3.**. Despite values in VFX-feed treatments were much lower than those observed in VFX-water treatments (i.e. maximum VFX concentration = 189 µg kg^{-1} dw in liver; **Figures 4.1.2.** and **4.1.3.**), tissues' VFX bioaccumulation followed a similar pattern, with the liver also being the primary organ for the bioaccumulation of this contaminant regardless of treatment (e.g. in VFX-feed treatment: 26.7± 86.5 µg kg^{-1} dw equivalent to an average NAR of 3.7 µg kg^{-1} day^{-1}; $p < 0.05$), followed by the brain, whereas much lower VFX concentrations were found in plasma and muscle (**Figures 4.1.4.A** and **4.1.5.A**). In addition, under the reference temperature and pCO_2 conditions, liver:plasma concentration ratio was also significantly higher than those observed in the brain and muscle at day 28 (10.7 ± 5.3; $p < 0.05$; **Figure 4.1.4.C**). As observed in VFX-water treatments, significant and negative correlations were also found between biometric data and VFX NARs in fish plasma (r = -0.67 and -0.72 for W and TL, respectively; $p < 0.05$), but not in the three fish tissues (**Table 4.1.3.**).

As for the effects of seawater temperature and pCO_2, overall, warming (with or without the combination of acidification) promoted a significant increase in fish W and TL compared to the reference temperature and pCO_2 conditions ($p < 0.05$; **Table 4.1.2.**). In parallel, similar to what was observed in VFX-water treatments, increased temperature and pCO_2 levels, acting alone or in combination, resulted in significantly lower VFX concentrations and NARs in the liver at day 28 (i.e. between 55.8 and 78.2 µg kg^{-1} dw, equivalent to average NARs between 1.7 and 2.0 µg kg^{-1} day^{-1} in Acid+VFX-feed and Warm+VFX-feed treatments; $p < 0.05$; **Figures 4.1.4.A** and **4.1.5.A**). Conversely, significantly higher VFX bioaccumulation was also observed in fish brain when warming acted alone (80.5 ± 2.0 µg kg^{-1} dw, equivalent to a NAR of 2.8 µg kg^{-1} day^{-1} in treatment Warm+VFX-feed; $p < 0.05$), but not when acidification was combined (i.e. Acid+Warm+VFX-feed; **Figures 4.1.4.A** and **4.1.5.A**). Furthermore, the lower VFX tissue concentrations along with enhanced VFX plasma concentrations under warming and acidification (see also **Annex 5, Table A.5.4.**), overall, decreased the tissue to plasma concentration ratios, particularly when both stressors were combined (**Figure 4.1.4.C**).

After the 7 days of elimination phase, distinct patterns were observed according to fish tissue and treatment (**Figures 4.1.4.B** and **4.1.5.B**). Fish exposed to warming and acidification exhibited lower VFX elimination in the liver, particularly when both abiotic stressors were combined (9.1 ± 3.0% in treatment Acid+Warm+VFX-feed) compared to those exposed to the reference conditions ($p < 0.05$; **Figure 4.1.5.B**). As for the brain and muscle, no significant differences were observed between treatments simulating the reference and increased seawater temperatures (i.e. between VFX-feed and Warm+VFX-feed and Acid+Warm+VFX-feed; $p > 0.05$), with EF values reaching up to 45.6% in the brain, and up to 100% in the muscle (**Figures 4.1.4.B** and **4.1.5.B**).

Conversely, acidification alone (i.e. Acid+VFX-feed) resulted either in very low VFX elimination (14.7 ± 4.4% in the liver), or in no elimination at all (in the remaining tissues; $p < 0.05$; **Figures 4.1.4.B** and **4.1.5.B**). Regarding tissue:plasma concentration ratios, significant differences between days 28 and day 35 were only found in liver of Acid+VFX-feed and Acid+Warm+VFX-feed treatments, as well as in muscle of VFX-feed treatment ($p < 0.05$; **Figure 4.1.4.C**).

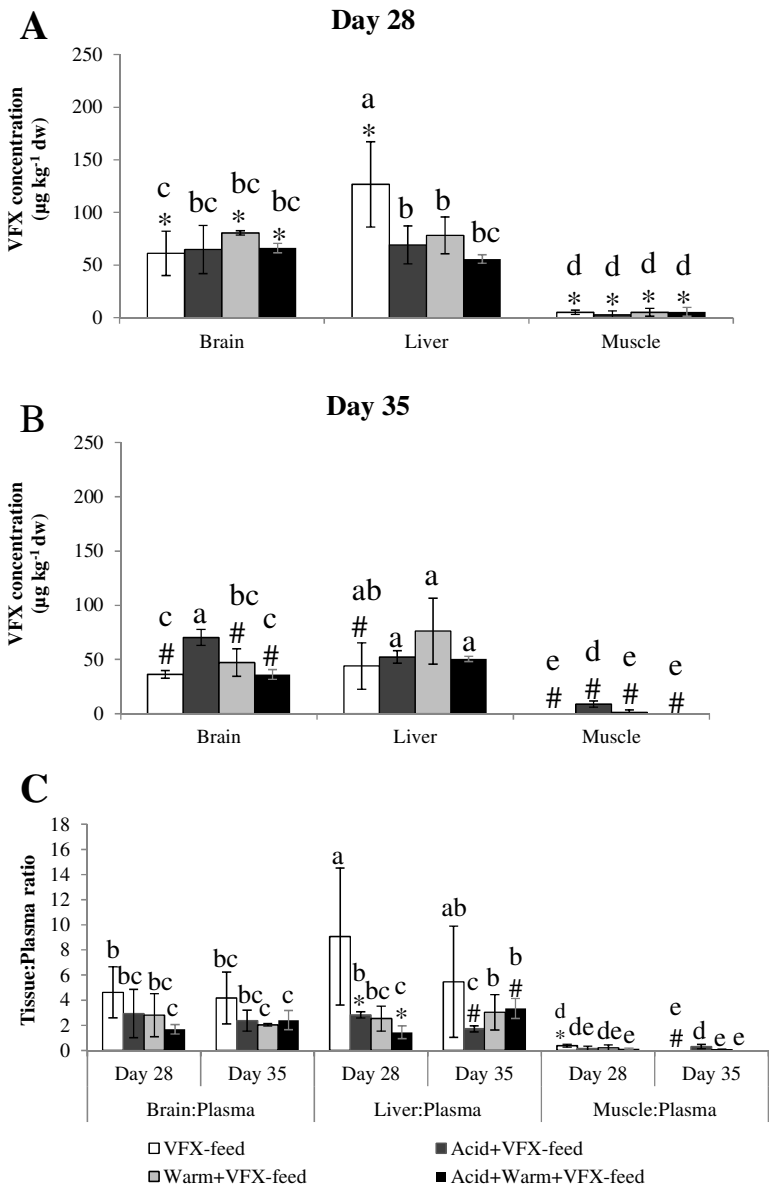

Figure 4.1.4. Venlafaxine (VFX) concentrations (µg kg^{-1} dw) in tissues of fish exposed to VFX via feed at day 28 (exposure phase; A) and day 35 (elimination phase; B), as well as, tissue to plasma VFX concentration ratio at days 28 and 35 (4C). Different letters indicate significant differences between tissues and treatments, whereas different symbols (* or #) indicate significant differences between day 28 and day 35 for the same tissue ($p < 0.05$). Abbreviations: Acid – simulated acidification (i.e. pCO_2 ~1500 µatm, equivalent to pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC).

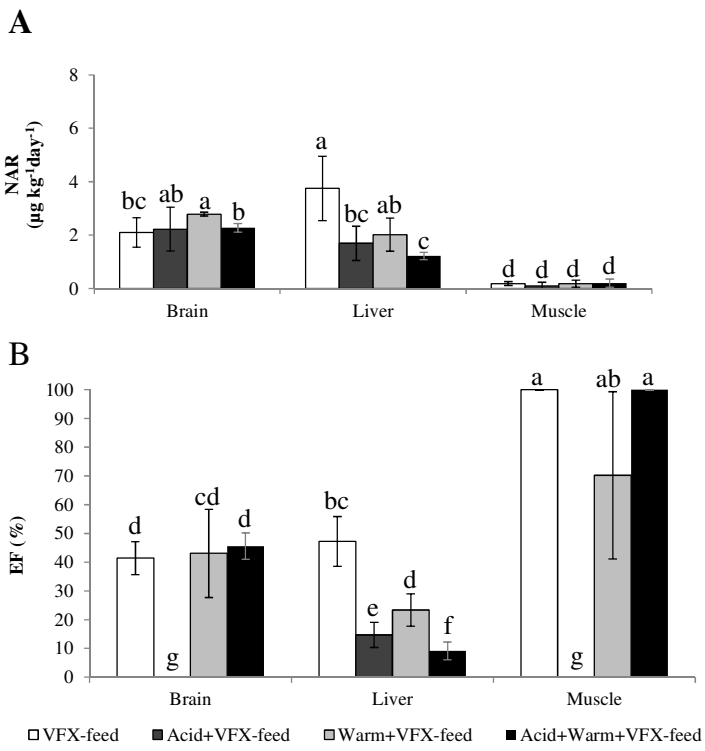

Figure 4.1.5. Venlafaxine (VFX) net accumulation rates at day 28 (NAR; µg kg^{-1} day^{-1}; A) and VFX elimination factors at day 35 (EF; %; B) in VFX-feed treatments. Different letters indicate significant differences among VFX-feed treatments ($p < 0.05$). Abbreviations: Acid – simulated acidification (i.e. pCO_2 ~1500 µatm, equivalent to pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC).

4. Discussion

4.1. VFX levels in feed, seawater and non-contaminated fish samples

VFX was not detected in the CTR feed, indicating that no external contamination occurred during feed preparation. Furthermore, these results assured that seawater was the only route of VFX exposure in VFX-water treatments during the first 28 days of trial, as well as that VFX dietary exposure did not occur during the elimination phase in all treatments (see **Table 4.1.1.**).

As for seawater samples, the results obtained evidenced that: i) no other source of contamination occurred in VFX-feed treatments, apart from the intended dietary VFX exposure; ii) VFX concentrations were steady in VFX-water treatments throughout the 28 days of exposure phase; and iii) the 25% of daily water renewal carried out in each incubating tank during the last 7 days of trial (i.e. elimination phase) diminished VFX concentrations in seawater, but did not enable a complete removal of this compound in VFX-water treatment. Furthermore, it should also be noted that VFX levels found in seawater samples from VFX-water treatments at day 35, might also be partially due to the potential desorption of VFX from fish skin and tank glass walls during the clearance phase (previously adsorbed during the exposure phase). Finally, VFX was not

detected in CTR feed nor in seawater samples from the Control treatment, but was still quantified at low levels in brain and liver composite samples of non-contaminated fish samples (**Table 4.1.1.**), suggesting that an external contamination might have occurred at some point, most likely during sample collection, processing and/or analysis due to contaminated materials (e.g. sample recipients, tools) and/or equipment.

4.2. VFX exposure route, tissue distribution, uptake and elimination

To the best of author's knowledge, there are no studies available concerning other VFX exposure pathways apart from water (e.g. Brooks, 2014; Zenker et al., 2014). Nevertheless, uptake mechanisms apart from contaminant passive diffusion across the gills (i.e. inhalation) can be particularly important in the case of PPCPs: these compounds are highly ionisable and cover a wide range of molecular polarity and, therefore, other specific biochemical mechanisms (e.g. ion trapping, protein binding) should also be accounted in terms of compound uptake and bioavailability (Klosterhaus et al., 2013, Stott et al., 2015). Furthermore, dietary exposure can have a preponderant role on contaminants' bioaccumulation, especially in marine predatory species with long life cycles, such as *A. regius* (Dijstra et al., 2013; Brooks et al., 2014; Zenker et al., 2014; Maulvault et al., 2016).

The two exposure routes led to different VFX tissue concentrations and NAR; i.e. NAR in VFX-water treatments was up to 50 times higher than in VFX-feed treatments (see **Figures 4.1.2.-4.1.5.**). This discrepancy can be attributed to the different VFX concentrations that were selected for water and feed exposure and, as previously mentioned, such selection was based on: i) VFX exposure via feed: VFX levels commonly found in species inhabiting contaminated coastal areas, susceptible to accumulate this contaminant, and that are natural preys of juvenile meagre (Martínez Bueno et al., 2014; Álvarez-Muñoz et al., 2015), as well as the VFX exposure levels which were expected to elicit the bioaccumulation of this compound to detectable levels during the timeline of the trials; and ii) VFX exposure via water: the lowest VFX concentration range previously reported to cause significant behavioural effects following short-term VFX exposure (Bisesi Jr et al., 2014). Thus, it should be noted that the present results should be looked at as a proof of concept, since the exposure water concentration is above values usually found in water bodies, and that VFX uptake patterns may depend on exposure dose and time (Brooks, 2014; Zenker et al., 2014; Silva et al., 2016). Furthermore, the present experimental design did not allow to investigate other parameters (e.g. respiration and ingestion rates, compound bioavailability), which would be crucial to draw conclusions regarding the predominance of one exposure route over the other. Yet, a recent multi-trophic marine study, reported that dietary exposure did not have a preponderant role compared to other exposure pathways, on the uptake of the antidepressants sertraline and fluoxetine (also weak basic pharmaceuticals; Boström et al., 2017).

When it comes to contaminant uptake, the favouring of one exposure route over another can be closely linked to the physical-chemical properties of the compound, particularly to its octanol-water partition coefficient (log KoW; Qiao et al., 2000). In general, compounds that have a log KoW < 3 have been pointed out to be mostly uptaken through inhalation, whereas those with log KoW > 6 are mostly uptaken through ingestion (e.g. Heath, 1995; Qiao et al., 2000). However, for ionisable compounds like VFX, log DoW instead of log KoW should be considered in order to account for the ionization of the compound in its partition. Venlafaxine has a log DoW of 1.78 at pH 8, which is lower than its log KoW (2.74-3.30; Aryal et al., 2012), suggesting that this compound can rather be uptaken by inhalation than ingestion. Nevertheless, this parameter cannot be used alone to explain the differences observed in the bioaccumulation of the different exposure routes since other physical-chemical properties can affect the uptake (Huerta et al. 2016). Thus, the present results also point out that VFX has the potential to be biomagnified by fish, i.e. to be transferred from food to biota (i.e. dietary exposure through the ingestion of a contaminated prey or a part of it), and such biomagnifying potential, along with biochemical and behavioural alterations in marine biota after chronic exposure to antidepressants (e.g. Valenti et al., 2012; Bisesi Jr. et al., 2014; Maulvault et al., 2018a), emphasize the ecological hazards they can pose to marine ecosystem.

Concerning VFX tissue partitioning, results showed that, regardless of exposure pathway, liver was the primary tissue for the uptake of VFX (see **Figures 4.1.2.- 4.1.5.**); this organ is responsible for the metabolization and redistribution of xenobiotics to other tissues, through the blood circulation (Wang et al., 2013; Maulvault et al., 2016). A similar pattern of compound partitioning in fish tissues and plasma (see **Figures 4.1.2.- 4.1.5.**, as well as **Annex 5, Table A.5.4.**) was also reported in previous studies focusing on VFX exposure in fish species (i.e. VFX concentration: liver > brain > muscle, in brook trout, *Salvelinus fontinalis*; Lajeunesse et al., 2011; VFX concentrations: liver > brain > plasma > muscle, in rainbow trout, *Oncorhynchus mykiss*; Grabicova et al., 2014), as well as on other common antidepressants (citalopram, sertraline and fluoxetine: liver > brain > plasma and muscle, in rainbow trout; Grabicova et al., 2014;). In general, higher BCF values were observed in the present study (water exposure; **Figure 4.1.3.B**) compared to those previously reported (Lajeunesse et al., 2011; Grabicova et al., 2014). For instance, in the recent study of Grabicova et al. (2014) performed on juvenile rainbow trout, the BCF values obtained after 13 days of exposure to VFX (330 ng L^{-1}) were below 6 L kg^{-1} in fish muscle, and up to 63 L kg^{-1} in liver. Similarly, lower VFX BCFs (between 2 and 18 L kg^{-1}) were also calculated in brook trout exposed to municipal wastewaters (VFX concentration ~40 ng L^{-1}) for a period of 3 months (Lajeunesse et al., 2011). Yet, the BCF obtained following 28 days of water exposure suggest that VFX is not bioaccumulative in fish tissues, based on the thresholds set by regulatory authorities (i.e. BCF > 1000 L kg^{-1} ww; EPA, 2012; or BCF > 2000 L kg^{-1} ww;

EU, 2011), and such results are consistent with previous findings (e.g. Lajeunesse et al., 2011; Grabicova et al., 2014; Serra-Compte et al., 2018). On the other hand, considering the different experimental conditions among studies, yielding discrepant BCF values, it can be hypothesized that the uptake mechanisms of antidepressants, such as VFX, may not only differ according to species (including possible differences between fish sizes, gender and other ecological features), but also be dependent on the exposure dose and time (e.g. Brooks et al., 2014; Zenker et al., 2014; Silva et al., 2016). Given the current lack of empirical data on this matter, further research should be carried out particularly considering different exposure concentrations and times, in order to better understand the toxicokinetics of these pharmaceuticals in the marine environment.

When assessing the bioconcentration/bioaccumulation of chemical contaminants, animal size and/or lipid content are two aspects that should also be considered, as these variables are often correlated with contaminants' tissue concentrations (Dijstra et al., 2013; Zenker et al., 2014; Maulvault et al., 2016). Indeed, the present results revealed that, after 28 days of VFX exposure via water, VFX bioconcentration was strongly (and negatively) correlated to animal size (except in fish muscle; see **Table 4.1.3.**), indicating that VFX concentration, in general, tends to decrease in fish tissues and plasma as the animal size increases, most likely due to somatic growth dilution (Dijstra et al., 2013). On the other hand, the absence of a significant correlation between fish size and tissue bioaccumulation in VFX-feed treatments (except in plasma) may be related to the exposure dose (i.e. the final concentration in VFX-enriched feed), as well as to the distinct bioavailability mechanisms of this compound according to the exposure route (Sanchez and Meier, 1997; Brooks et al., 2014; Gray and Hughes, 2015).

Finally, regarding the elimination phase, the present results showed a very low persistence of VFX in the muscle of fish exposed via feed, partially due to the much lower bioaccumulation found in this tissue (see **Figures 4.1.4.-4.1.5.**). Furthermore, low persistence was also observed in the liver of VFX-feed fish, which is in agreement with the following facts that liver is a primary organ for contaminant metabolization and its subsequent detoxification (Aryal et al., 2012; Maulvault et al., 2016, 2017). In contrast, no elimination was observed in fish tissues exposed to VFX via water under the reference temperature and pCO_2 conditions (see **Figures 4.1.2.-4.1.3.**). Such results were most likely due to the high tissue concentrations reached in this treatment (in line with high water exposure concentration), as well as the presence of VFX in seawater during the elimination phase of the trial.

4.3. Effects of warming and/or acidification and the link with seafood safety

Despite the limited information about emerging contaminants in terms of ecotoxicology and food safety, recent studies suggest that abiotic variables (e.g. changes in temperature, pH, light

and dissolved oxygen) can play a key role on PPCPs' chemical behaviour, degradation, metabolization and toxicity (Farré et al., 2008; Burke et al., 2014; Qian et al., 2015).

In general, marine organisms subjected to warmer temperatures exhibit enhanced metabolism, accompanied by increased ventilation and feeding rates in response to higher metabolic demands. Such changes often translate into a higher uptake of contaminants dissolved in the water column (via respiration) or present in feeds or natural preys (via ingestion; e.g. Schiedek et al., 2007; Manciocco et al., 2014; Maulvault et al. 2016; Sampaio et al. 2016). For instance, a recent study showed that sotalol and sulfamethoxazole bioconcentration on *Mytillus galloprovincialis* did increase with water warming, but VFX bioconcentration actually decreased in the same warming conditions (Serra-Compte et al., 2018). Contrasting this trend, in the present work, warming generally resulted in lower VFX tissue uptake (regardless of exposure route), with the exception of fish brain from Warm+VFX-feed treatment (see **Figures 4.1.2.-4.1.5.**). Such decrease could be due to the fact that enhanced metabolic rates at higher temperature can also promote increased contaminant elimination rates, i.e. contaminant metabolization and subsequent excretion, thus translating in lower contaminant bioaccumulation in fish tissues (Schiedek et al., 2007; Manciocco et al., 2014; Maulvault et al. 2016; Sampaio et al. 2016). Noteworthy, the lower VFX uptake at higher temperatures (i.e. in the liver of VFX-feed and in brain and liver of VFX-water treatments) could also be related to the somatic growth dilution, given the higher W and TL (i.e. enhanced growth rates; see **Table 4.1.2.**) observed in treatments simulating seawater warming (e.g. Dijstra et al., 2013; Maulvault et al., 2016; Anacleto et al., 2018).

Concerning VFX elimination, warming (alone or combined with acidification) led to diminished VFX EF in the liver (a primary organ for metabolic breakdown of xenobiotics and subsequent transport to other organs and/or excretion) compared to the reference temperature conditions (see **Figure 4.1.5.B**), suggesting that the physiological stress induced by +5 ºC of temperature increase not only affected the excretion of VFX in its parental form, but also compromised its transformation into different metabolites at the liver. In agreement with this, in a previous study on juvenile *Dicenthrachus labrax* exposed to MeHg, liver's ability to detoxify this pollutant was also compromised under +4 ºC of temperature increase (Maulvault et al., 2016). Thus, the physiological and metabolic alterations elicited warming, which resulted in lower elimination of VFX (parent compound) in the liver, also reinforced the need to consider, in future studies, the effects of the surrounding abiotic conditions when assessing compound metabolization mechanisms.

Seawater acidification has also the potential to directly or indirectly influence marine species' physiology and metabolism, therefore, affecting the way biota cope with the presence of contaminants (Freitas et al. 2016; Sampaio et al., 2018). Since most PPCPs are highly ionisable, but cell membranes are usually very poorly permeable to charged (ionic) contaminant forms,

variations of the surrounding seawater pCO_2 levels are likely to influence the uptake and toxicity of this group of chemical contaminants (e.g. Orvos et al. 2002; Rowett et al. 2016; Serra-Compte et al., 2018). However, in this study VFX was mostly present positively charged under both pH 8.0 units and pH 7.6 units (VFX dissociation constant (pKa) = 9.6; Singh et al., 2008) and, therefore, physical-chemical properties of the compound would not be affected by pH. An increment in VFX bioaccumulation in brain and plasma (see **Figures 4.1.4.-4.1.5.** and *Table A.3*) was observed under lower pH levels (Acid+VFX-feed) compared to the reference pH conditions regardless of temperature (VFX-feed and Warm+VFX-feed), although a decrease in liver and muscle bioaccumulation was also observed. Thus, physiological alterations induced in fish exposed to this treatment may explain the observed variations (e.g. Rosa et al., 2016; Sampaio et al., 2016, 2018), as well as the possible damages elicited by hypercapnia in apical epithelial membranes of fish tissues, which can then facilitate contaminant penetration into cells (Freitas et al., 2016; Sampaio et al., 2016, 2018; Shi et al., 2016; Velez et al., 2016).

On the other hand, the altered physiological condition and metabolic activity promoted by lower pH levels may also justify the significantly lower VFX elimination in liver compared to reference conditions. Contrasting the present findings, Serra-Compte et al. (2018) observed an increment in VFX elimination in mussels under acidification conditions.

As for the combination of warming and acidification, results showed that one abiotic variable may: i) enhance the effects of the other, i.e. VFX elimination in liver further decreased in Warm+Acid+VFX-feed fish, in relation to Acid+VFX-feed and Warm+VFX-feed; or ii) reverse the effects of the other, i.e. higher VFX bioaccumulation was observed in the brain of fish exposed via feed (Warm+VFX-feed), yet such increase at higher temperatures was counteracted by acidification (i.e. in Warm+Acid+VFX-feed). A recent study performed on marine bivalves exposed to a broad range of emerging contaminants also reported increased bioaccumulation of dechlorane 602 in treatments simulating warming combined with acidification, compared to treatments simulating a single stressor (Maulvault et al., 2018b). In contrast, this study further revealed that the effects of warming and acidification acting alone on TBBPA and PFOS bioaccumulation were reversed when these stressors were combined. Following this trend, Sampaio et al. (2018) reported that reduced pH also countered MeHg increased bioaccumulation observed in juvenile meagre *A. regius* exposed to warming in isolation. In VFX water exposed treatments an opposite trend was observed in fish liver, where VFX elimination in Warm+Acid+VFX-water increased when compared to the reference temperature and pCO_2 conditions (**Figures 4.1.3.- 4.1.5.**). This difference suggests that: i) exposure route may have a stronger impact in VFX bioaccumulation and elimination mechanisms than the surrounding abiotic conditions; and ii) the different bioaccumulation and elimination patterns observed according to the exposure route may actually be dose dependent.

Finally, VFX has been detected in commercially important seafood species together with other PPCPs, although at relatively low levels (e.g. up to 36.1 ng g^{-1} dw in bivalve species; Álvarez-Muñoz et al., 2015; up to 16.4 ng g^{-1} dw in brown trout, *Salmo trutta* m. *fario* Grabicova et al., 2017). This raises several concerns from the human health perspective, given the potential hazards to seafood consumers when ingesting contaminated species. Thus, there is a reinforcement that these pollutants should not only be regulated in seafood species, but also be included in regular environmental monitoring campaigns. In addition, the risks of PPCP's human exposure should be estimated considering both the present climate conditions and those foreseen for the ocean of tomorrow. Even though the results do not point out to increased VFX uptake in fish tissues in a climate change context, the trends observed in this study confirmed that seawater abiotic variables can have a strong influence on PPCPs' bioaccumulation and toxicity by impairing biota's mechanisms of contaminant bioaccumulation, metabolization and elimination. Such findings highlight that careful attention should be given to the expected effects of climate change when establishing maximum permissible limits for emerging environmental contaminants, such as PPCPs, in seafood and when defining human exposure recommendations to this group of contaminants.

5. Conclusions

Overall, data confirmed that VFX can be uptaken by fish species through contaminated water and diet. Seawater temperature and pCO_2 levels can strongly affect VFX uptake and elimination in marine organisms, by affecting contaminant physical and chemical properties, as well as animal metabolism and physiological responses. In general, warming resulted in increased VFX uptake in brain (and plasma), most likely due to the enhanced organisms' metabolic rates. In parallel, the diminished VFX uptake in liver might be attributed to enhanced VFX elimination rates at higher temperatures. In contrast, acidification resulted not only in lower VFX uptake in the liver compared to the reference pH conditions, but also in diminished elimination in all fish tissues, most likely due to the physiological stress induced in fish leading to impaired metabolic rates. The results also showed that a stressor may enhance the effects of another (e.g. VFX elimination in fish liver was further decreased when warming and acidification acted together), as well as reverse it (i.e. the higher VFX uptake in fish brain under warming alone was counteracted by acidification). The distinct patterns observed in the different scenarios strengthened the need to carry out greater research efforts to understand how multiple environmental stressors in a climate change context, such as warming, acidification and pollution, interact with each other, as well as how to integrate these variables in future regulations and recommendations regarding both the ecological and human health impact.

Ethical statement

Fish trials were conducted according to legal regulations (EU Directive 2010/63), and approved by the Ethical Committee of the Faculty of Sciences of the Lisbon University, overseen by the Portuguese National Competent Authority (Direção-Geral de Alimentação e Veterinária, DGAV). All researchers and technicians involved in the maintenance, handling and sampling of live animals were certified in Laboratory Animal Sciences, by the Federation of European Laboratory Animal Science Associations (FELASA).

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Part 2.

Living in a multi-stressors environment: An integrated biomarker approach to assess the ecotoxicological response of meagre (*Argyrosomus regius*) to venlafaxine, warming and acidification

Manuscript 5.

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Abstract

Pharmaceuticals, such as the antidepressant venlafaxine (VFX), have been frequently detected in coastal waters and marine biota, and there is a growing body of evidence that these pollutants can be toxic to non-target marine biota, even at low concentrations. Alongside, climate change effects (e.g. warming and acidification) can also affect marine species' physiological fitness, consequently, compromising their ability to cope with the presence of pollutants. Yet, information regarding interactive effects between pollutants and climate change-related stressors is still scarce. Within this context, the present study aims to assess the differential ecotoxicological responses (antioxidant activity, heat shock response, protein degradation, endocrine disruption and neurotoxicity) of juvenile fish (*Argyrosomus regius*) tissues (muscle, gills, liver and brain) exposed to VFX (via water or feed), as well as to the interactive effects of warming (ΔT°C = +5 °C) and acidification (ΔpCO_2 ~ +1000 µatm, equivalent to ΔpH = -0.4 units), using an integrated multi-biomarker response (IBR) approach.

Overall, results showed that VFX toxicity was strongly influenced by the uptake pathway, as well as by warming and acidification. More significant changes (e.g. increases surpassing 100% in lipid peroxidation, LPO, heat shock response protein content, HSP70/HSC70, and total ubiquitin content, Ub,) and higher IBR index values were observed when VFX exposure occurred via water (i.e. average IBR = 19, against 17 in VFX-feed treatment). The co-exposure to climate change-related stressors either enhanced (e.g. glutathione S-transferases activity (GST) in fish muscle was further increased by warming) or attenuated the changes elicited by VFX (e.g. vitellogenin, VTG, liver content increased with VFX feed exposure acting alone, but not when co-exposed with acidification). Yet, increased stress severity was observed when the three stressors acted simultaneously, particularly in fish exposed to VFX via water (i.e. average IBR = 21). Hence, the distinct fish tissues responses elicited by the different scenarios emphasized the relevance of performing multi-stressors ecotoxicological studies, as such approach enables a better estimation of the environmental hazards posed by pollutants in a changing ocean and, consequently, the development of strategies to mitigate them.

Keywords: venlafaxine, fish, warming, acidification, integrated multi-biomarker response.

1. Introduction

The exhaustive exploitation of natural resources, along with the increasing production and release of pollutants into the environment, including the "so called" greenhouse gases (GHG, e.g. CO_2, CH_4, N_2O), have contributed to one of the greatest environmental concerns of our time. According to the latest report of the Intergovernmental Panel on Climate Change (IPCC, 2014), GHG emissions have reached unprecedented levels in the last 50 years, unequivocally causing the warming of the planet, with most of the energy produced in the form of heat being stored in the ocean (only ~1% of the total energy produced within the climate system is stored in the atmosphere). Furthermore, the increasing release of GHG has also resulted in a higher oceanic uptake of CO_2 (i.e. increased CO_2 partial pressure, pCO_2, which causes the drop of the average seawater pH), therefore, leading to a phenomenon known as "ocean acidification" (IPCC 2014; McNeil and Sasse, 2016). Thus, even if efforts are made to keep GHG emissions at today's rates and pollution levels stable in a short/medium term, recent projections indicate that, within a 50 to 100 years' timeframe, seawater temperature and pCO_2 levels will still increase as high as 5 ºC and 1000 µatm, respectively (IPCC 2014; McNeil and Sasse, 2016).

Climate change-related stressors can have negative impacts on marine species, affecting their physiology, metabolism and ecological fitness (e.g. Madeira et al., 2016, Rosa et al., 2014, 2016, 2017), thus, making them less resilient to the co-exposure with other environmental stressors, such as chemical contaminants (Sampaio et al., 2016, 2018; Maulvault et al., 2017; 2018a,b,c, Serra-Compte et al., 2018). On the other hand, changes of the surrounding abiotic conditions (e.g. temperature and pH) can also affect chemical contaminants' physical and chemical properties (i.e. speciation, transport, transfer among compartments), as well as their uptake, elimination and toxicity to marine organisms (e.g. Marques et al., 2010; Maulvault et al., 2016, 2017, 2018a, Sampaio et al., 2018; Serra-Compte et al., 2018). Yet, understanding the potential interactions between climate change-related stressors and pollution is a topic that only recently raised attention within the scientific community and, therefore, further research efforts are urgently required to better forecast the ecotoxicological implications of climate change.

Pharmaceuticals and personal care products (PPCPs) comprise a wide diversity of non-regulated compounds of emerging concern, including human and veterinary pharmaceuticals, cosmetics, preservatives, detergents, among others. These compounds have been frequently detected in coastal waters (concentrations ranging from ng L^{-1} up to mg L^{-1}; e.g. Gaw et al., 2014; Arpin-Pont et al., 2016; Rodriguez-Mozaz et al., 2017), as well as in marine biota (e.g. Vandermeersch et al., 2015; Rodriguez-Mozaz et al., 2017), promoting several adverse effects in non-target organisms (Best et al., 2014; Bisesi Jr. et al., 2014; Bidel et al., 2016; Maulvault et al., 2018b,c). Despite recent evidence suggested that PPCPs' bioavailability and toxicity is strongly mediated by the surrounding abiotic conditions (e.g. Gul et al., 2015; Rowett et al., 2016; Serra-

Compte et al., 2018), their ecotoxicological implications to marine organisms under future climate conditions are still ununderstood.

With the aim of better understanding the interactions between PPCPs exposure and abiotic variables, we have recently conducted two studies focused on the widely used psychiatric pharmaceutical venlafaxine (VFX), using juvenile meagre (*Argyrosomus regius*) as model organism (Maulvault et al., 2018a,b). Both of these studies constituted relevant proofs of concept, confirming that the co-exposure to abiotic conditions not only affected VFX's bioaccumulation and elimination mechanisms in fish species (Maulvault et al., 2018a), but also accentuated the behavioural impairments elicited by VFX (Maulvault et al., 2018b). Such findings arose the interest for conducting a third study to assess the potential biochemical alterations at tissue/cell level induced by exposure to VFX, warming and/or acidification. In this context, the present study aimed to investigate the ecotoxicological responses (antioxidant enzymes activities, chaperoning and protein degradation, neurotoxicity and endocrine disruption) of juvenile *A. regius* tissues (muscle, gills, liver and brain) after 28 days of co-exposure to VFX (via water, i.e. [VFX] ~20 µg L^{-1}, and via feed, i.e. [VFX] ~160 µg kg^{-1} dry weight, dw), warming (ΔTºC = +5 ºC) and acidification (ΔpCO_2 ~ +1000 µatm, equivalent to ΔpH = -0.4 units), using an integrated multi-biomarker response (IBR) approach.

2. Materials and Methods

2.1. Acclimation

A. regius specimens were reared until the juvenile stage at the aquaculture pilot station of the "Portuguese Institute for the Sea and Atmosphere (EPPO-IPMA, Olhão, Portugal)" using routine hatchery conditions, and were subsequently transported to the aquatic facilities of "Laboratório Maritimo da Guia (MARE-FCUL, Cascais, Portugal)". Here, fish with similar morphometry (total length: 6.8 ± 0.6 cm; total weight 2.6 ± 0.8 g; n = 120) were randomly and equitably distributed in 30 rectangular shaped incubating glass tanks (10 treatments x 3 replicate tanks; each tank with 50 L of total volume; **Figure 4.2.1.**) within independent recirculation aquaculture systems (RAS). To maintain seawater quality and abiotic parameters at the adequate levels, each tank was equipped with: i) protein skimmer (Reef SkimPro, TMC Iberia, Portugal); ii) UV disinfection (Vecton 300, TMC Iberia, Portugal); iii) biological filtration (model FSBF 1500, TMC Iberia, Portugal); iv) chemical filtration (activated carbon, Fernando Ribeiro Lda, Portugal); v) automatic seawater refrigeration systems (± 0.1 °C; Frimar, Fernando Ribeiro Lda, Portugal) and submerged digital thermostats (200W, V2Therm, TMCIberia, Portugal) to control seawater temperature; and vi) individual pH probes (GHL, Germany) connected to a computerized pH control system (± 0.1 pH units; Profilux 3.1N, GHL, Germany) to monitor seawater pH in each tank (measures every 2 seconds), and adjust to the adequate levels whenever

needed, through the injection of either CO_2 (Air Liquide, Portugal; to decrease pH) or CO_2-filtered air (Stella 200 air pumps, Aqua One Pro, Aqua Pacific UK Ltd, United Kingdom; to increase pH) via submerged air stones displayed in each tank. As routine procedures, every day, fish faeces were cleaned and 25% of seawater total volume was renewed in each tank. Ammonia, nitrite and nitrate levels were checked every week using colorimetric tests (Tropic Marin, USA), and kept below detectable levels, with the exception of nitrates (i.e. kept below 2.0 mg L^{-1}). Furthermore, seawater total alkalinity was measured in every tank on a weekly basis, following the protocol described by Sarazin et al. (1999), and the combination of total alkalinity (AT) and pH was used to calculate carbonate system parameters (average values obtained for each treatment can be consulted in **Annex 6, Table A.6.1.**).

Before beginning the trial, fish were acclimated for a period of 30 days, under the following abiotic conditions: i) dissolved oxygen (DO) > 5 mg L^{-1}; ii) temperature (T ºC) = 19.0 ± 0.5 °C; iii) pH = 8.00 ± 0.10 units; iv) salinity = 35 ± 1 ‰; and v) photoperiod = 12 hours light:12 hours darkness. Temperature, pH, salinity and DO were checked daily using a multi-parameter measuring instrument (Multi 3420 SET G, WTW, Germany). During acclimation, fish from all treatments were fed ~2% of their body weight (bw) with a non-contaminated fish diet, i.e. with CTR feed (VFX concentration in CTR feed < 0.30 ng g^{-1}, i.e. < the limit of detection, LOD, of the methodology used to determine VFX concentrations in feed samples; Gros et al., 2012; Maulvault et al., 2018a). Details regarding feeds preparation and VFX determination were previously presented in Maulvault et al. (2018a,b). Moreover, feeds nutritional composition is available in **Annex 6, Table A.6.2**. No mortality was observed during the acclimation period (nor during the trial).

2.2. Experimental setup

The experimental setup used to expose fish to VFX (via diet or water), warming and acidification was similar to the ones previously described by Maulvault et al. (2018a,b). Briefly, the following experimental conditions (acting in isolation or combined) were simulated: i) either the absence of VFX contamination (i.e. non-contaminated treatments) or exposure to this pollutant through two different pathways (VFX uptake from water via inhalation, i.e. VFX-water treatments, or VFX uptake from diet via ingestion, i.e. VFX-feed treatments); and ii) either the temperature and pCO_2 conditions normally used in juvenile meagre rearing conditions in Southern Europe (i.e. 19 ºC and ~500 µatm pCO_2, equivalent to 8.0 pH units) or the projected seawater warming (i.e. Warm treatments; ΔTºC = +5 ºC) and acidification (i.e. Acid treatments; ΔpCO_2 ~+1000 µatm, equivalent to ΔpH = -0.4 units).

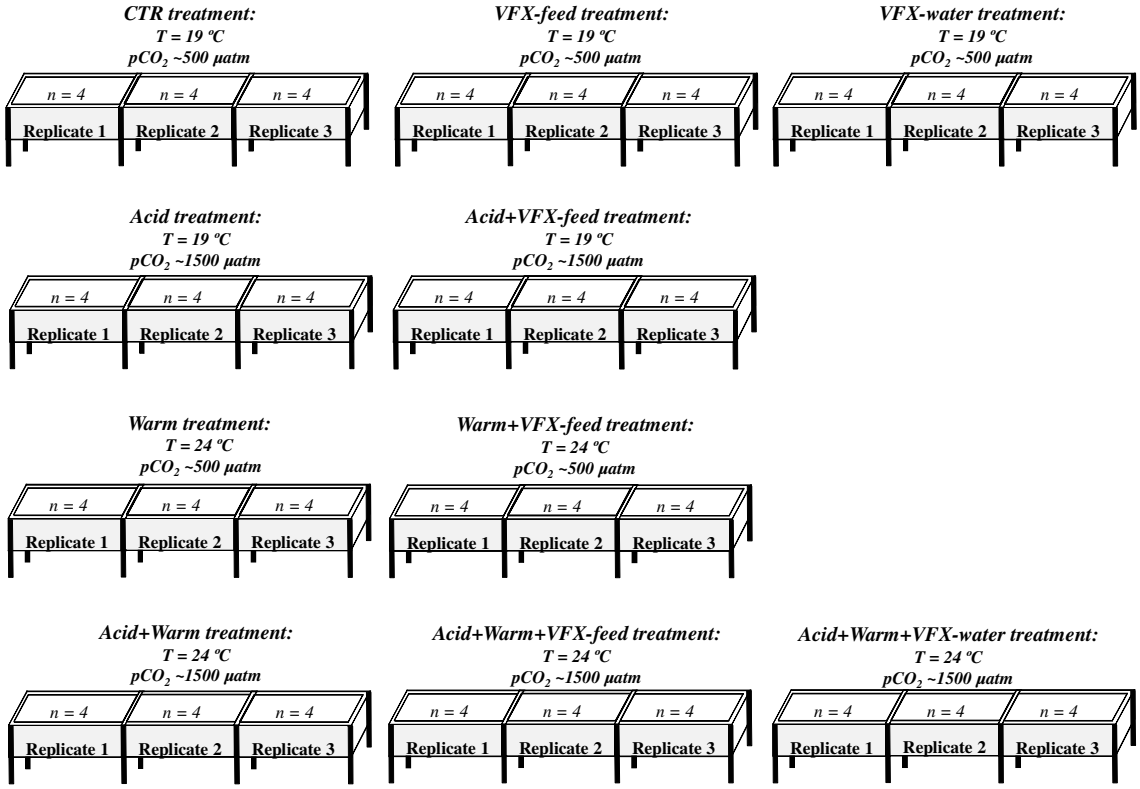

Figure 4.2.1. Experimental setup. Abbreviations: CTR – reference temperature and pH conditions (i.e. T = 19 ºC and pH = 8.0 units); VFX-feed – VFX exposure via feed; VFX-water – VFX exposure via water; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC).

To simulate VFX feed exposure, a VFX-enriched diet (with the same nutritional composition as CTR feed; see **Annex 6, Table A.6.1.**) was prepared by top-coating fish CTR feed pellets with a VFX hydrochloride stock solution ($C_{17}H_{27}NO_2 \cdot HCl$, >98%, Sigma-Aldrich; solubilized in deionized water; detailed description of VFX-enriched feed preparation presented in Maulvault et al., 2018a,b). VFX final concentration in VFX-enriched feed was 161.7 ± 17.1 µg kg^{-1} dry weight (dw). To simulate VFX water exposure, a VFX hydrochloride stock solution was also prepared to daily spike each tank, achieving a final VFX concentration of 20.2 ± 3.8 µg L^{-1} in each tank (in a steady state). The criteria followed to select such concentrations, as well as data regarding the stability assessment of VFX concentrations in both feed and seawater throughout the trial are shown in Maulvault et al. (2018a,b).

To simulate seawater warming and acidification, one week before initiating the trial, seawater temperature and pH were gradually adjusted (+1 ºC and -0.1 pH unit per day), until reaching 24 ºC and ~1500 µatm pCO_2 (equivalent to pH = 7.6 units) in tanks simulating climate change conditions (**Figure 4.2.1.**), according to the projections of the Intergovernmental Panel for Climate Change (scenario RCP8.5; IPCC, 2014), as well as considering the intervals of future CO_2 amplification scenarios described by McNeil and Sasse (2016). Due to experimental limitations, only VFX dietary exposure was selected to investigate all possible interactions

between stressors, in a full cross-factorial design (i.e. Warm+VFX-feed, Acid+VFX-feed and Warm+Acid+VFX-feed treatments; the criteria used to prioritize VFX-feed exposure, over VFX-water exposure, was previously described in Maulvault et al., 2018a,b). Nevertheless, the effect of warming and acidification acting together (i.e. the worst-case scenario) was assessed in treatments simulating both VFX exposure routes (i.e. seawater and dietary exposures; Acid+Warm+VFX-water and Acid+Warm+VFX-feed; **Figure 4.2.1.**).

In summary, ten treatments were carried out (n = 4 animals per replicate tank, i.e. 12 fish per treatment; the experimental setup is shown in **Figure 4.2.1.**) during 28 days of trial, i.e. 4 non-contaminated treatments in which fish were exposed to the corresponding temperature and pCO_2 conditions while being daily fed (2% bw) with CTR feed (CTR, Acid, Warm and Acid+Warm treatments), 4 treatments simulating VFX dietary exposure, in which fish were exposed to the corresponding temperature and pCO_2 conditions while being daily fed (2% bw) with VFX-enriched feed (VFX-feed, Acid+VFX-feed, Warm+VFX-feed and Acid+Warm+VFX-feed treatments), and 2 treatments simulating VFX water exposure, in which fish were exposed to seawater daily spiked with a VFX stock solution, as well as to the corresponding temperature and pCO_2 conditions, while being daily fed (2% bw) with CTR feed.

2.3. Samples collection

After 28 days of trial, 6 fish were randomly collected from each treatment (i.e. 2 fish collected from each of the 3 replicate tanks that composed each treatment), euthanized by immersion in an overdosed MS222 solution (2000 mg L^{-1}; Sigma-Aldrich, USA) buffered with sodium bicarbonate (1 g of $NaHCO_3$ to 1 g of MS222 in 1 L of seawater) for 10 min. Euthanized fish were dissected and fish muscle, gills, liver and brain tissues were collected. Each tissue (approximately 100 mg of muscle, gills and liver, and about 40 mg of brain) was individually homogenized in ice-cold conditions with 1.0 mL of phosphate buffered saline (PBS; 140 mM NaCl, 3mM KCl, 10 mM KH_2PO_4, pH = 7.40 ± 0.02; reagents from Sigma-Aldrich, Germany), using an Ultra-Turrax® device (T25 digital, Ika, Germany). Crude homogenates were centrifuged in 1.5 mL microtubes for 15 minutes at 10.000 g and 4 ºC. Supernatants were then transferred to new microtubes, immediately frozen and kept at -80 ºC until further analyses.

2.4. Integrated ecotoxicological response of *A. regius*

To assess fish tissue responses to VFX, warming and acidification exposure, eight ecotoxicological biomarkers (of exposure and/or effect) were selected, each corresponding to distinct biological endpoints:

i) Antioxidant defences – catalase activity [CAT; spectrophotometric enzymatic assay adapted from Johansson and Borg (1988)], superoxide dismutase activity [SOD; spectrophotometric enzymatic assay adapted from Sun et al. (1988)] and glutathione S-transferases activity [GST; spectrophotometric enzymatic assay adapted from Habig et al. (1974)];

ii) Cellular damage – Lipid peroxidation [(LPO); measured as the total malondialdehyde (MDA) content through the thiobarbituric acid test, adapted from Uchiyama and Mihara (1978)];

iii) Protein chaperoning / Heat shock response – HSP70/HSC70 content [determined through an enzyme-linked immunosorbent assay (ELISA), based on the methodology described by Njemini et al. (2005)];

iv) Protein degradation / DNA repair – Ubiquitin content [Ub; determined through the ELISA methodology, as described by Madeira et al. (2014)];

v) Reproduction / Endocrine disruption – Vitellogenin liver content [VTG; determined through the ELISA methodology based on the methodology described by Denslow et al. (1999)];

vi) Neurotoxicity – Acetylcholinesterase activity [AChE; spectrophotometric enzymatic assay adapted from Ellman et al. (1961)].

These biochemical biomarkers have been widely employed in ecotoxicological studies, being considered as reliable and suitable to assess the effects of xenobiotics exposure, including antidepressants (e.g. Fong and Ford, 2014; Rodrigues et al., 2014; Ding et al., 2017), as well as of climate change-related effects (e.g. Rosa et al., 2016; Maulvault et al., 2017, 2018c; Sampaio et al., 2018). To normalize the results of each biomarker (i.e. results expressed in mg of protein), total protein levels were also quantified in each sample according to the Bradford assay (Bradford, 1976). Furthermore, to facilitate data consultation and interpretation, biomarker results are presented throughout as U mg^{-1} protein, with the exception of SOD for which values were presented as % of inhibition (all biomarker units can be consulted in **Annex 6, Methodologies**). All biomarker assays were carried out using reagents of pro analysis grade or higher, as well as 96-well microplates from Nunc-Roskilde (Denmark) and a microplate reader (Multiskan Go 1510, ThermoFisher Scientific, USA). Further details regarding the methodologies used to determine tissue ecotoxicological responses are available in **Annex 6, Methodologies**. Each sample was analysed in triplicate.

In order to integrate the various ecotoxicological responses, the integrated multi-biomarker response (IBR) was calculated for each treatment and tissue, according to the methodology proposed by Beliaeff and Burgeot (2002), later modified by Guerlet et al. (2010). Further details regarding the IBRs calculations are presented in **Annex 6, Methodologies**. As IBR compares

biomarker responses of organisms exposed to stressors with those of animals under control conditions, in general, lower biomarker scores (and, thus, lower IBR index values) indicate a better health status (higher animal fitness), whereas higher scores usually indicate that organisms are in a poorer physiological condition (i.e. stressed; e.g. Ferreira et al., 2015; Madeira et al., 2016, 2018; Maulvault et al., 2018c). To compare *A. regius* physiological state from a whole organism perspective, the average IBR value for each treatment (using values from all tissues) was also calculated (Madeira et al., 2016). Star plots and IBR calculations were performed using Microsoft Excel software.

2.5. Statistical analyses

As standard procedure, data were first tested for normality and homoscedasticity through Kolmogorov–Smirnov and Levene tests, respectively. Data were log or square-rooted transformed, whenever at least one of these assumptions was not verified. To evaluate the presence of significant differences between treatments in biomarker levels, nested factorial ANOVAs were carried out, using replicate tank as nesting factor, and tissue (brain, liver and muscle) and/or treatment as variables. Moreover, to determine the existence of significant differences in IBRs between treatments (all tissues combined), a simple one-way ANOVA analysis was performed instead. After performing the ANOVA analyses, post-hoc Tukey HSD tests were conducted to identify significant differences. Statistical analyses were performed at a significance level of 0.050, using STATISTICA™ software (Version 7.0, StatSoft Inc., USA).

3. Results

3.1. Biomarker tissue levels

Tissue biomarker responses in *A. regius* exposed to the different treatments are shown in **Figures 4.2.2.-4.2.4.** (biomarker values, i.e. activity/concentration, in CTR treatment can also be consulted in **Annex 6, Table A.6.3.**). Diminished CAT activity was generally observed in the muscle of fish exposed to VFX (regardless of exposure route), acidification and/or warming in relation to CTR treatment (i.e. decreases ranging from 31% in Acid and Warm treatments up to 68% in Acid+Warm+VFX-water treatment; $p < 0.050$), with the exception of VFX-water and Acid+Warm+VFX-feed treatments in which no significant changes were observed ($p > 0.050$; **Figure 4.2.2.A** and **Table 4.2.1.**). In contrast, CAT activity was significantly enhanced by VFX exposure in fish liver (i.e. VFX-feed and VFX-water treatments) and by warming in brain, regardless of pCO_2 conditions and VFX exposure (i.e. CAT activity Warm, Warm+VFX-feed, Acid+Warm, Acid+Warm+VFX-feed and Acid+Warm+VFX-water treatments; $p < 0.050$; **Figures 4.2.2.C,D** and **Table 4.2.1.**). As for fish gills, only VFX-feed and Acid+Warm+VFX-

water treatments significantly affected CAT activity in this tissue, the first treatment inhibiting this enzyme's activity (52% decrease; $p < 0.050$), and the second enhancing it (56%; $p < 0.050$; **Figure 4.2.2.B** and **Table 4.2.1.**). In general, all stressors diminished SOD activity at least in one tissue, yet, such effect was particularly notorious in Acid+Warm+VFX-water treatment, as all studied tissues showed significantly higher inhibition compared to CTR treatment (maximum SOD inhibition obtained in fish brain, corresponding to 21% decrease in relation to CTR treatment; $p < 0.050$; **Figures 4.2.2.E-H** and **Table 4.2.1.**). Except for acidification alone, all stressors significantly increased GST activity in fish muscle, with the highest value being found in treatments simulating VFX water exposure (i.e. >100 increase in VFX-water and Acid+Warm+VFX-water treatments; $p < 0.050$; **Figure 4.2.2.I** and **Table 4.2.1.**). Similarly, GST brain activity was also significantly enhanced by warming alone or combined with the other two stressors (though the highest value was found in Warm treatment, corresponding to an increase of >100% in relation to CTR treatment; $p < 0.050$), as well as by the combination of acidification plus VFX via feed (i.e. 93% increase in Acid+VFX-feed treatment in relation to CTR treatment; $p < 0.050$; **Figure 4.2.2.L** and **Table 4.2.1.**). In contrast, a significant inhibition of this enzyme's activity was observed in the liver of fish from VFX-water (60%), Acid (63%), Acid+VFX-feed (36%), Warm (26%) and Acid+Warm (57%) treatments ($p < 0.050$; **Figure 4.2.2.K** and **Table 4.2.1.**). All stressors (with the exception of VFX-feed exposure alone) and their interactions significantly increased total MDA concentrations compared to CTR treatment, with tissue LPO being particularly significant in fish gills and brain regardless of treatment (i.e. MDA gill content increased ~100% in all treatments, with the exception of VFX-feed treatment; $p < 0.050$; **Figures 4.2.2.M-P** and **Table 4.2.1.**). Noteworthy, the highest LPO value was found in fish gills from Acid+Warm+VFX-feed treatment (i.e. 0.062 ± 0.003 U mg proteín^{-1}), corresponding to an average increase of 345% in relation to CTR treatment (**Figure 4.2.2.N** and **Table 4.2.1.**).

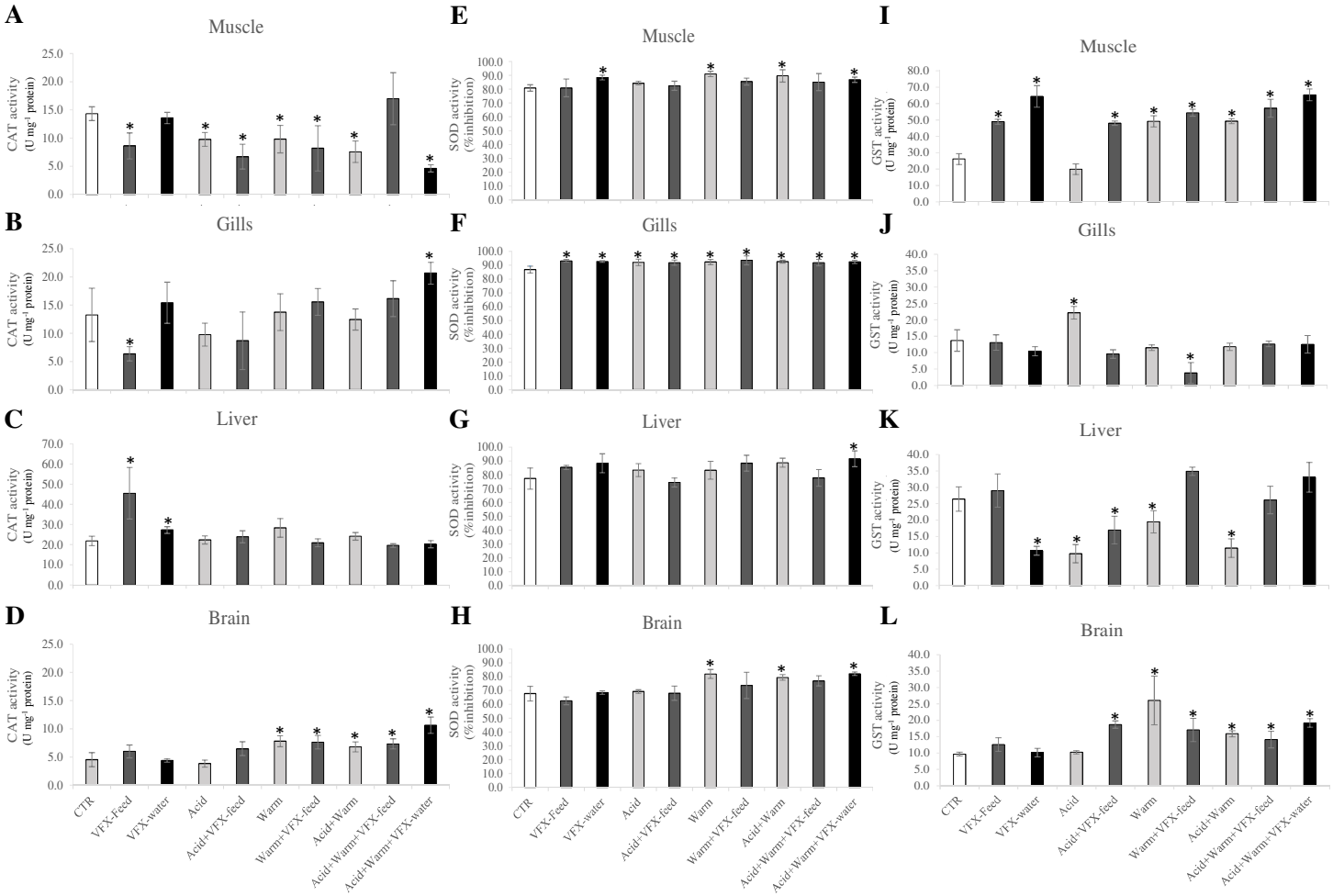

Figure 4.2.2. Antioxidant enzymes activity (GST, U mg^{-1} protein; CAT, U mg^{-1} protein; SOD, % inhibition) and lipid peroxidation (LPO, U mg^{-1} protein) in muscle (A, E, I, M), gills (B, F, J; N), liver (C, G, K; O) and brain (D, H, L, P) in *A. regius* (average ± standard deviation; $n = 6$) after 28 days of exposure to the different experimental conditions. Asterisk indicate significant differences between CTR and the remaining treatments ($p < 0.05$).

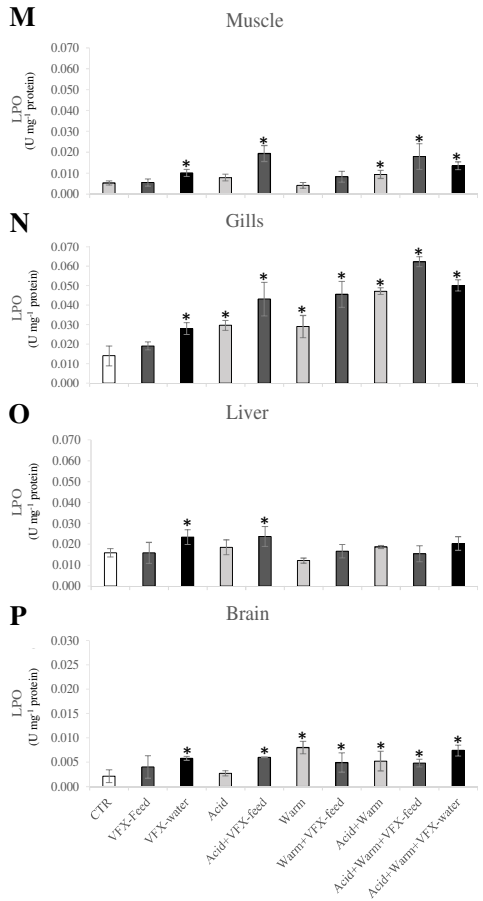

Figure 4.2.2. ***(continuation)*** Antioxidant enzymes activity (GST, U mg^{-1} protein; CAT, U mg^{-1} protein; SOD, % inhibition) and lipid peroxidation (LPO, U mg^{-1} protein) in muscle (A, E, I, M), gills (B, F, J; N), liver (C, G, K; O) and brain (D, H, L, P) in *A. regius* (average ± standard deviation; $n = 6$) after 28 days of exposure to the different experimental conditions. Asterisk indicate significant differences between CTR and the remaining treatments ($p < 0.05$). Abbreviations: CTR – reference temperature and pH conditions (i.e. T = 19 ºC and pH = 8.0 units); VFX-feed – VFX exposure via feed; VFX-water – VFX exposure via water; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC).

Table 4.2.1. Percentages of change (%) induced by VFX (via feed or water) exposure, acidification and warming in relation to the CTR treatment, as well as, statistical comparisons (Tukey HSD test) between CTR and the other treatments (i.e. *p-values*; n = 6 individuals analysed per treatment). "↑" before the value indicates a significant increase compared to values found in CTR treatment, whereas "↓"indicates a significant decrease ($p < 0.05$).

		CAT		SOD		GST		LPO		HSP70/HSC70		Ub		VTG		AChE	
		%	*p-value*	*%*	*p-value*	*%*	*p-value*	*%*	*p-value*	*%*	*p-value*	*%*	*p-value*	*%*	*p-value*	*%*	*p-value*
VFX-Feed	**Muscle**	**↓39.8**	0.024	NS	>0.050	**↑87.3**	<0.001	NS	>0.050	NS	>0.050	**↑51.8**	<0.001	-		-	-
	Gills	**↓52.1**	0.040	**↓7.3**	<0.001	NS	>0.050	NS	>0.050	NS	>0.050	NS	>0.050	-		-	-
	Liver	**↑108.3**	<0.001	NS	>0.050	NS	>0.050	NS	>0.050	NS	>0.050	**↑148.8**	0.001	**↑122.1**	<0.001	-	-
	Brain	NS	>0.050	NS	>0.050	NS	>0.050	NS	>0.050	**↑334.1**	<0.001	NS	>0.050	-		**↑37.2**	0.004
VFX-water	**Muscle**	NS	>0.050	**↓9.3**	0.011	**↑146.1**	<0.001	**↑90.3**	0.008	NS	>0.050	NS	>0.050	-		-	-
	Gills	NS	>0.050	**↓6.8**	<0.001	NS	>0.050	**↑99.5**	0.003	**↑145.3**	<0.001	**↑115.6**	0.001	-		-	-
	Liver	**↑25.0**	0.032	NS	>0.050	**↓59.8**	<0.001	**↑47.6**	0.032	**↑36.5**	0.020	**↑202.4**	<0.001	NS	>0.050	-	-
	Brain	NS	>0.050	NS	>0.050	NS	>0.050	**↑169.9**	0.006	**↑627.6**	<0.001	**↑253.6**	0.008	-		NS	>0.050
Acid	**Muscle**	**↓31.8**	0.033	NS	>0.050	NS	>0.050	NS	>0.050	NS	>0.050	NS	>0.050	-		-	-
	Gills	NS	>0.050	**↓6.1**	0.003	**↑62.1**	<0.001	**↑11.6**	0.002	NS	>0.050	**↑180.0**	<0.001	-		-	-
	Liver	NS	>0.050	NS	>0.050	**↓63.2**	<0.001	NS	>0.050	**↑140.0**	<0.001	**↑144.3**	0.001	**↓57.3**	0.004	-	-
	Brain	NS	>0.050	NS	>0.050	NS	>0.050	NS	>0.050	**↑268.5**	<0.001	**↑104.8**	0.035	-		**↑39.7**	0.002
Acid+VFX-feed	**Muscle**	**↓53.2**	0.001	NS	>0.050	**↑83.6**	<0.001	**↑264.2**	<0.001	NS	>0.050	**↑46.5**	0.003	-		-	-
	Gills	NS	>0.050	**↓5.8**	0.005	NS	>0.050	**↑207.5**	<0.001	NS	>0.050	NS	>0.050	-		-	-
	Liver	NS	>0.050	NS	>0.050	**↓35.8**	0.005	**↑49.3**	0.023	NS	>0.050	NS	>0.050	NS	>0.050	-	-
	Brain	NS	>0.050	NS	>0.050	**↑93.3**	<0.001	**↑179.6**	0.003	**↑391.9**	<0.001	**↑104.0**	0.028	-		**↑89.9**	<0.001
Warm	**Muscle**	**↓31.4**	0.042	**↓12.5**	0.004	**↑88.3**	<0.001	NS	>0.050	**↑35.9**	0.026	**↑76.3**	<0.001	-		-	-
	Gills	NS	>0.050	**↓6.4**	0.002	NS	>0.050	**↑107.1**	0.002	NS	>0.050	NS	>0.050	-		-	-
	Liver	NS	>0.050	NS	>0.050	NS	>0.050	NS	>0.050	**↑81.4**	<0.001	**↑101.8**	0.011	**↑46.6**	0.021	-	-
	Brain	**↑73.4**	0.001	**↓21.0**	<0.001	**↑169.6**	<0.001	**↑73.8**	<0.001	**↑203.6**	0.013	NS	>0.050	-		**↑168.3**	<0.001
Warm+VFX-feed	**Muscle**	**↓42.8**	0.011	NS	>0.050	**↑108.1**	<0.001	NS	>0.050	NS	>0.050	NS	>0.050	-		-	-
	Gills	NS	>0.050	**↓7.8**	<0.001	**↑72.4**	<0.001	**↑224.9**	<0.001	NS	>0.050	NS	>0.050	-		-	-
	Liver	NS	>0.050	NS	>0.050	NS	>0.050	NS	>0.050	**↓48.2**	<0.001	NS	>0.050	**↑122.0**	<0.001	-	-
	Brain	**↑69.2**	0.001	NS	>0.050	**↑76.5**	<0.001	**↑130.3**	0.005	**↑358.6**	<0.001	NS	>0.050	-		**↑75.7**	<0.001
Acid+Warm	**Muscle**	**↓47.1**	0.004	**↓10.8**	0.019	**↑88.6**	<0.001	**↑77.1**	0.029	NS	>0.050	**↑33.6**	0.025	-		-	-
	Gills	NS	>0.050	**↓6.6**	<0.001	NS	>0.050	**↑236.3**	0.001	**↓46.2**	0.033	NS	>0.050	-		-	-
	Liver	NS	>0.050	NS	>0.050	**↓56.7**	<0.001	NS	>0.050	**↑70.7**	<0.001	**↑108.0**	0.023	NS	>0.050	-	-
	Brain	**↑51.2**	0.036	**↓17.2**	0.004	**↑64.6**	<0.001	**↑144.6**	0.031	**↑138.2**	<0.001	NS	>0.050	-		**↑172.9**	<0.001

Table 4.2.1. ***(continuation)*** Percentages of change (%) induced by VFX (via feed or water) exposure, acidification and warming in relation to the CTR treatment, as well as, statistical comparisons (Tukey HSD test) between CTR and the other treatments (i.e. *p-values*; $n = 6$ individuals analysed per treatment). "↑" before the value indicates a significant increase compared to values found in CTR treatment, whereas "↓"indicates a significant decrease ($p < 0.05$). Abbreviations: CAT – catalase activity; SOD – superoxide dismutase activity; GST – glutathione S-transferase activity; LPO - lipid peroxidation measured as the MDA concentration; HSP70/HSC70 - heat shock proteins concentration; Ub - total ubiquitin concentration; VTG – vitellogenin concentration; AChE – acetylcholinesterase activity; NS – No significant alteration ($p > 0.05$) compared to the CTR treatment; VFX-feed – VFX exposure via feed; VFX-water – VFX exposure via water; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC).

		CAT		**SOD**		**GST**		**LPO**		**HSP70/HSC70**		**Ub**		**VTG**		**AChE**	
		%	***p-value***	***%***	***p-value***	***%***	***p-value***	***%***	***p-value***	***%***	***p-value***	***%***	***p-value***	***%***	***p-value***	***%***	***p-value***
Acid+Warm+VFX-feed	**Muscle**	NS	>0.050	NS	>0.050	↑**118.5**	<0.001	↑**236.6**	<0.001	NS	>0.050	NS	>0.050	-		-	-
	Gills	NS	>0.050	↓**5.8**	0.005	NS	>0.050	↑**343.9**	<0.001	NS	>0.050	NS	>0.050	-		-	-
	Liver	NS	>0.050	NS	>0.050	NS	>0.050	NS	>0.050	↓**70.5**	<0.001	↑**127.2**	0.004	NS	>0.050	-	-
	Brain	↑**62.6**	0.004	NS	>0.050	↑**46.0**	0.011	↑**125.4**	<0.001	↑**706.1**	<0.001	NS	>0.050	-		↑**67.7**	<0.001
Acid+Warm+VFX-water	**Muscle**	↓**67.7**	<0.001	↓**7.3**	0.016	↑**149.9**	<0.001	↑**154.0**	<0.001	↑**46.2**	0.015	↓**35.4**	0.049	-		-	-
	Gills	↑**55.7**	0.022	↓**6.4**	0.001	NS	>0.050	↑**257.4**	<0.001	↑**>100**	<0.001	NS	>0.050	-		-	-
	Liver	NS	>0.050	↓**18.5**	0.006	NS	>0.050	NS	>0.050	↓**24.0**	0.026	NS	>0.050	↓**53.6**	0.023	-	-
	Brain	↑**136**	<0.001	↓**21.2**	<0.001	↑**98.3**	<0.001	↑**245.9**	<0.001	↑**246.1**	0.001	NS	>0.050	-		↑**159.0**	<0.001

HSP70HSC70 and Ub contents are presented in **Figure 4.2.3**. Protein chaperoning and degradation was overall induced by the exposure to the three stressors (alone or combined), with the brain of fish from Acid+Warm+VFX-feed treatment revealing the highest HSP70/HSC70 content (i.e. 5.16 ± 0.92 µg mg proteín^{-1}, i.e. 706% increase in relation to CTR; **Figures 4.2.3.A-D** and **Table 4.2.1.**), whereas higher Ub contents were found when stressors acted individually (muscle: 0.26 ± 0.01 µg mg proteín^{-1} in Warm treatment, i.e. 76% increase; gills: 0.21 ± 0.05 µg mg proteín^{-1} in Acid treatment, i.e. 180% increase; liver and brain: 0.23 ± 0.05 µg mg proteín^{-1} and 0.12 ± 0.06 µg mg proteín^{-1}, respectively, i.e. 202% and 254% increases, respectively, both in VFX-water treatment; $p < 0.050$; **Figures 4.2.3.E-H** and **Table 4.2.1.**). Noteworthy, in fish muscle, HSP70/HSC70 synthesis was only significantly induced in Warm (36% increase; $p < 0.050$) and Acid+Warm+VFX-water (46% increase; $p < 0.050$) treatments (**Figures 4.2.3.A-D** and **Table 4.2.1.**). Yet, some exceptions were also observed, i.e. significant HSP70/HSC70 synthesis inhibition was observed when warming was combined with acidification (i.e. 46% reduction in gills from Acid+Warm treatment) and/or VFX via feed or water (i.e. 48%, 71% and 24% reduction in liver from Warm+VFX-feed, Acid+Warm+VFX-feed and Acid+Warm+VFX-water, respectively; $p < 0.050$; **Figures 4.2.3.A-D** and **Table 4.2.1.**). Moreover, Ub synthesis was also impaired in fish muscle Acid+Warm+VFX-water treatment (i.e. 35% reduction in VFX relation to CTR treatment; $p < 0.050$; **Figure 4.2.3.E** and **Table 4.2.1.**).

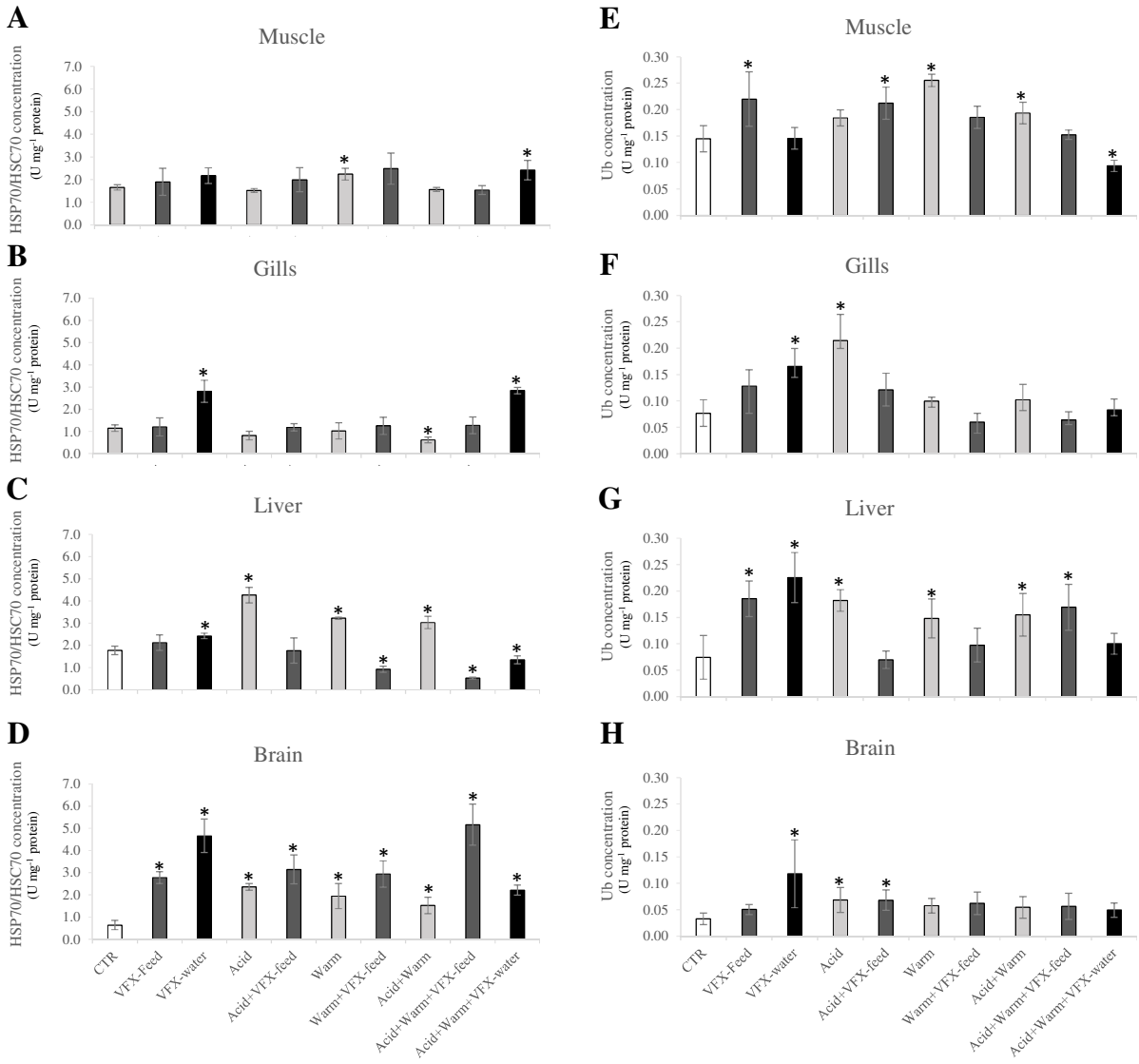

Figure 4.2.3. Heat shock proteins (HSP70/HSC70; A-D) and total ubiquitin (Ub; E-H) concentrations (U mg^{-1} protein; average ± standard deviation; n = 6) in muscle (A, E), gills (B, F), liver (C, G) and brain (D, H) in *A. regius* after 28 days of exposure to the different experimental conditions. Asterisk indicate significant differences between CTR and the remaining treatments ($p < 0.05$). Abbreviations: CTR – reference temperature and pH conditions (i.e. T = 19 ºC and pH = 8.0 units); VFX-feed – VFX exposure via feed; VFX-water – VFX exposure via water; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC).

VTG liver content and AChE brain activity are shown in **Figure 4.2.4**. VTG contents in the liver of *A. regius* were significantly increased by warming and VFX exposure via feed acting alone or combined (i.e. 47% increase in Warm treatment and 122% increase in VFX-feed and Warm+VFX-feed treatments in relation to CTR treatment; $p < 0.050$), whereas acidification alone or combined with warming and VFX via water significantly inhibited the production of this protein (i.e. 57% and 54% in Acid and Acid+Warm+VFX-water treatments, respectively, in relation to CTR treatment; $p < 0.050$; **Figure 4.2.4.A** and **Table 4.2.1.**). All treatments significantly enhanced AChE activity in the brain, apart from VFX-water treatment, though it is

worth mentioning that warming alone or combined with acidification and/or VFX-water exposure yielded the highest enzyme activity (i.e. 168%, 173% and 159% increases in Warm, Acid+Warm and Acid+Warm+VFX-water treatments, respectively; **Figure 4.2.4.B** and **Table 4.2.1.**).

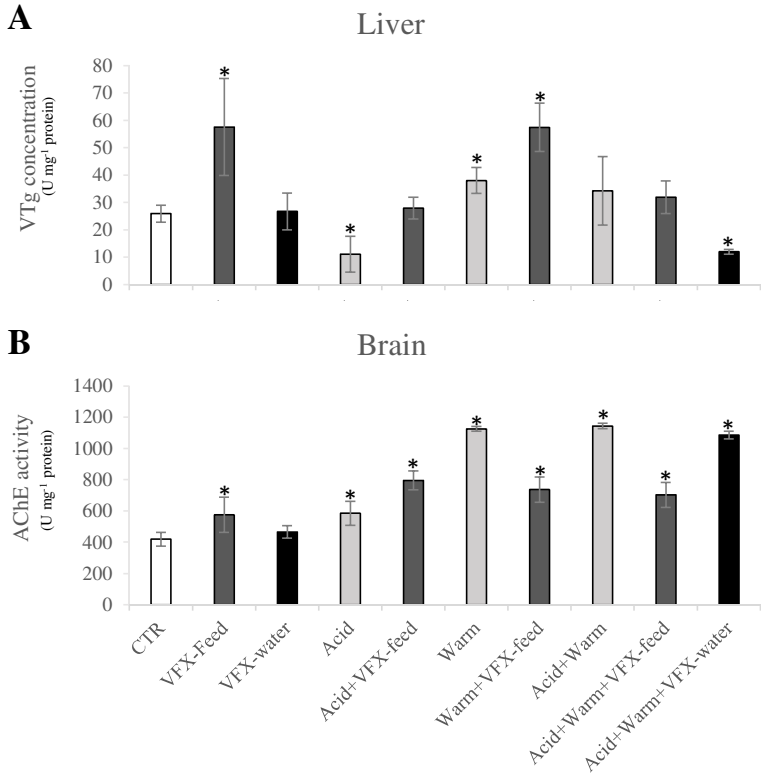

Figure 4.2.4. Vitellogenin liver concentration (VTG; A) and acetylcholinesterase brain activity (AChE, B) in A. regius (U mg^{-1} protein; average ± standard deviation; $n = 6$) after 28 days of exposure to the different experimental conditions. Asterisk indicate significant differences between CTR and the remaining treatments ($p < 0.05$). Abbreviations: CTR – reference temperature and pH conditions (i.e. T = 19 ºC and pH = 8.0 units); VFX-feed – VFX exposure via feed; VFX-water – VFX exposure via water; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC).

3.2. Integrated biomarker responses (IBRs)

IBR index values for each tissue and treatment and the corresponding starplots are shown in **Figure 4.2.5.** (individual scores of each biomarker in the different fish tissues and treatments can also be consulted shown in **Annex 6, Table A.6.4.**). In general, CTR samples presented lower biomarker scores than those obtained in the remaining treatments (differing in ≥ 0.5 units) and, thus, lower total IBR indexes were always found in this treatment (with the exception of muscle from Acid treatment, which revealed a value similar to CTR samples), regardless of tissue (**Figure 4.2.5.** and **Annex 6, Table A.6.4.**). Differences between CTR and the other treatments were further confirmed through the *One-Way ANOVA* analysis combining the total IBR index of all tissues (*One-way ANOVA* results*: MS* = 85.47; *F* = 5.98 and $p < 0.001$; **Figure 4.2.5.A**).

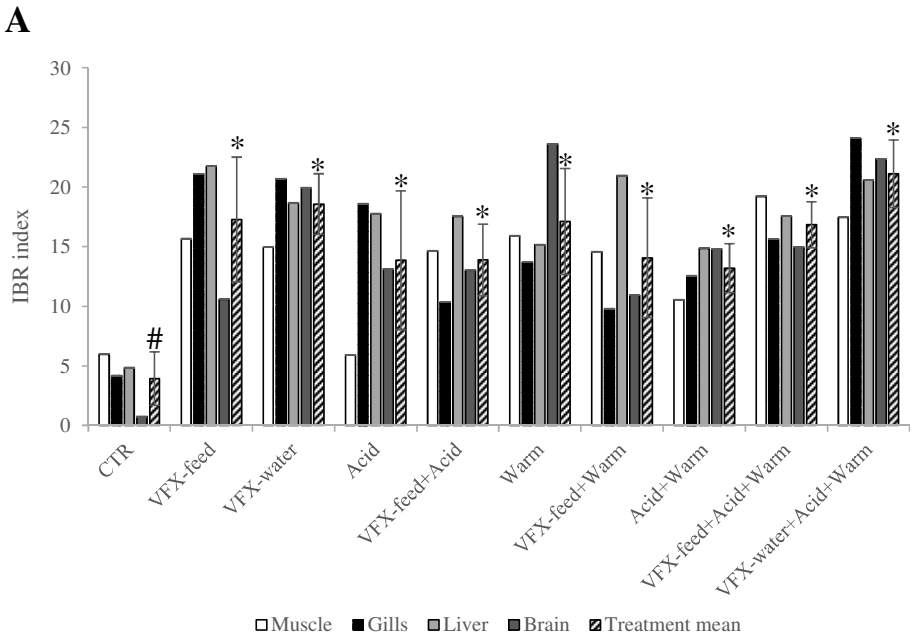

Figure 4.2.5. IBR index values for each tissue/treatment (A) and star plots of biomarker scores in the different fish tissues (muscle: B – E; gills: F– I; liver: J-M; brain: N – Q). Different symbols (# or *) indicate significant differences between the mean IBR of each treatment (i.e. average IBR value of the 4 tissues analysed; $p < 0.05$).

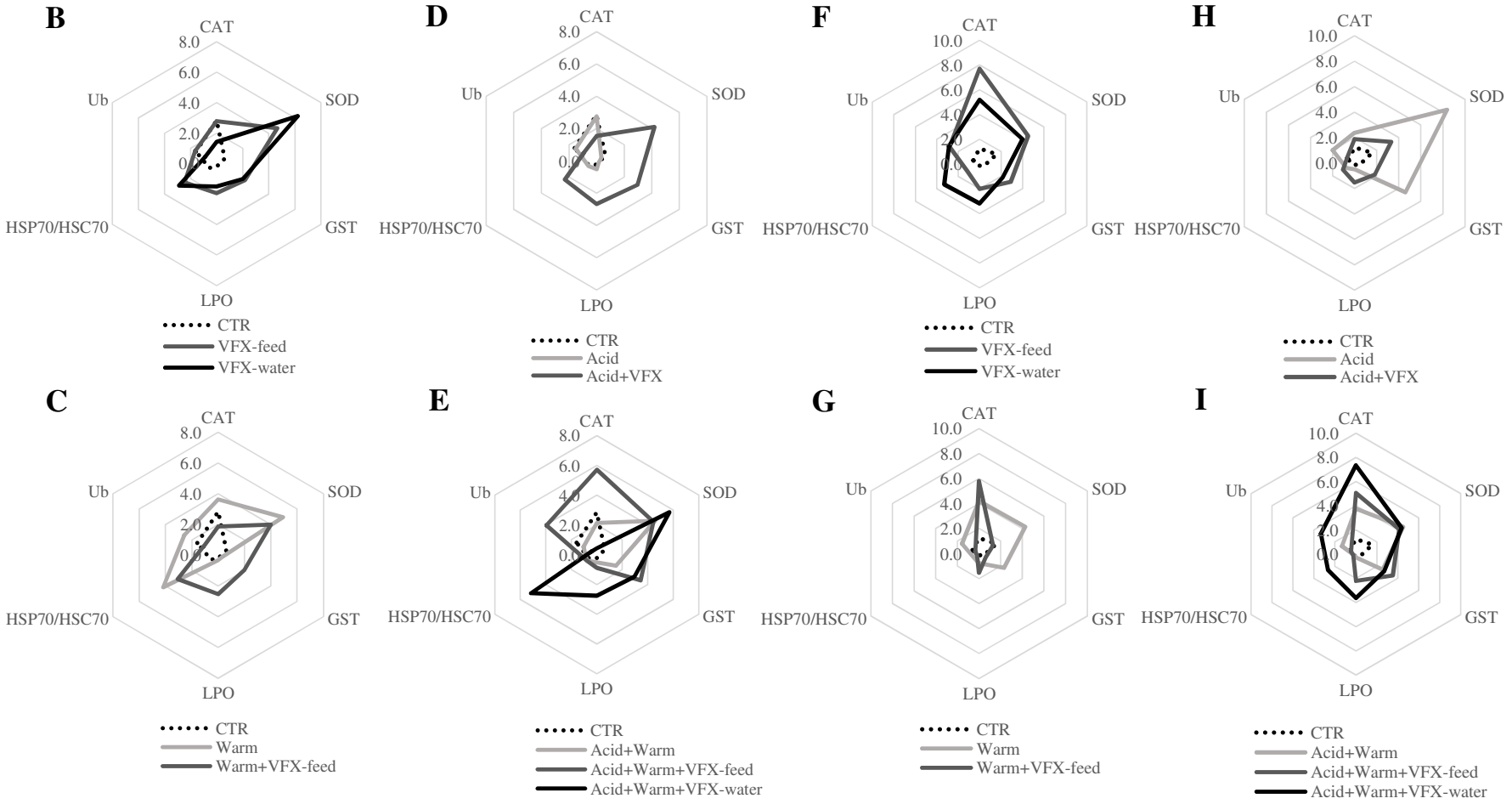

Figure 4.2.5. ***(continuation)*** IBR index values for each tissue/treatment (A) and star plots of biomarker scores in the different fish tissues (muscle: B – E; gills: F– I; liver: J-M; brain: N – Q). Different symbols (# or *) indicate significant differences between the mean IBR of each treatment (i.e. average IBR value of the 4 tissues analysed; $p < 0.05$).

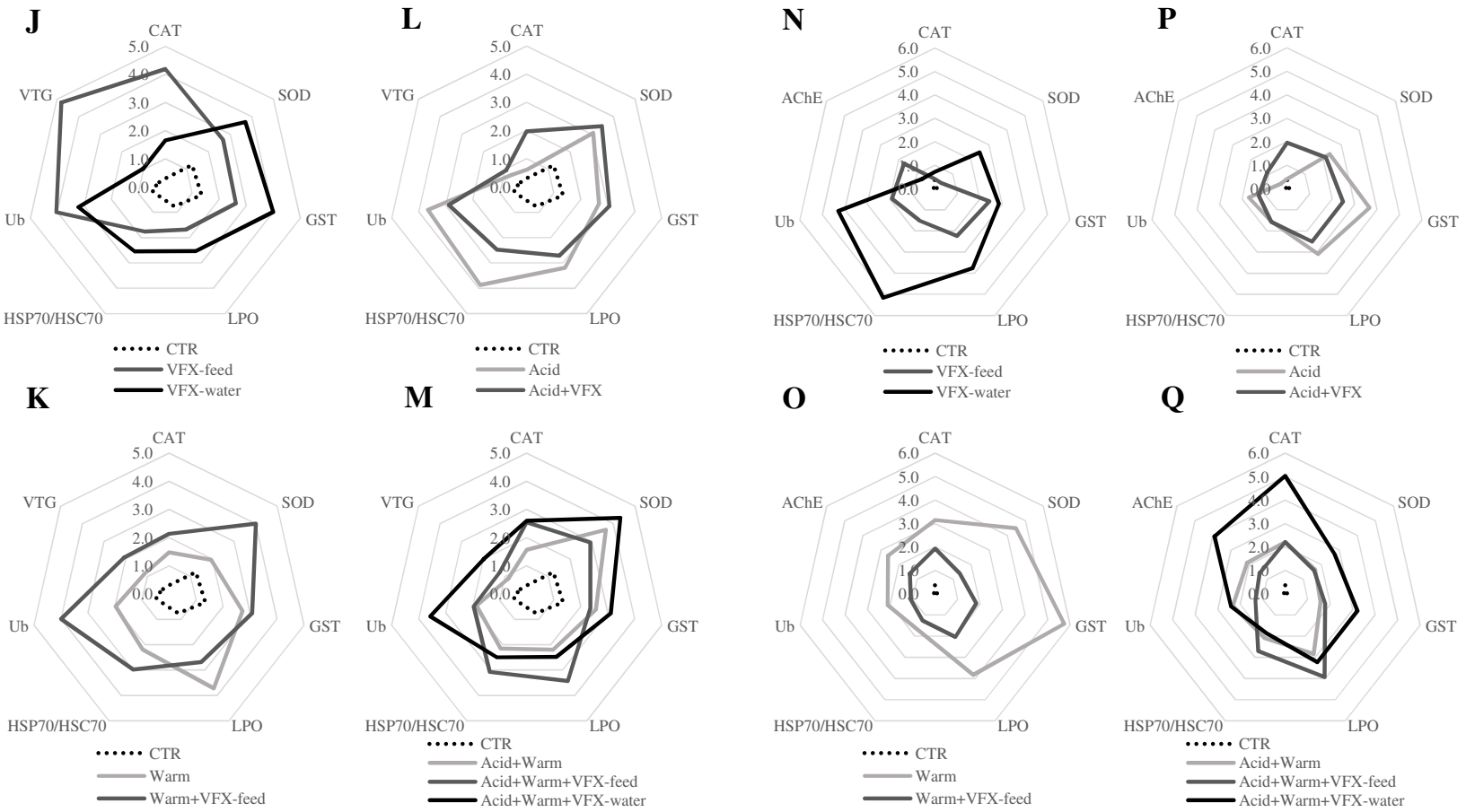

Figure 4.2.5. ***(continuation)*** IBR index values for each tissue/treatment (A) and star plots of biomarker scores in the different fish tissues (muscle: B – E; gills: F– I; liver: J-M; brain: N – Q). Different symbols (# or *) indicate significant differences between the mean IBR of each treatment (i.e. average IBR value of the 4 tissues analysed; $p < 0.05$). Abbreviations: CAT – catalase activity; SOD – superoxide dismutase activity; GST – glutathione S-transferase activity; LPO - lipid peroxidation, measured as MDA concentration; HSP70/HSC70 - heat shock proteins concentration; Ub - total ubiquitin concentration; VTG – vitellogenin concentration; AChE – acetylcholinesterase activity; CTR – reference temperature and pH conditions (i.e. T = 19 ºC and pH = 8.0 units); VFX-feed – VFX exposure via feed; VFX-water – VFX exposure via water; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC).

In contrast, no significant differences among tissues (all treatments combined) were observed (*One-way ANOVA* results*: MS* = 27.75; *F* = 0.69 and *p* = 0.563; **Figure 4.2.5.A**).

Looking at the variations according to exposure route, VFX-feed treatment revealed slightly higher total IBR index values than VFX-water treatment in fish muscle (i.e. 16 against 15, respectively) and liver (i.e. 22 against 19, respectively), whereas the opposite trend was observed in the brain (i.e. 11 against 20, respectively; **Figure 4.2.5.A**). In fish gills, similar values were yielded by the two VFX exposure routes (i.e. around 21; **Figure 4.2.5.A**). Combining the responses of all tissues, higher mean IBR index value was obtained in VFX-water treatment (i.e. 19) compared to VFX-feed treatment (i.e. 17; **Figure 4.2.5.A**).

Regarding the effect of abiotic stressors (acidification and warming acting separately), in fish muscle, acidification acting alone yielded the lowest IBR value (i.e. IBR = 6), while similar values were found in Acid+VFX-feed, Warm and Warm+VFX-feed treatments (i.e. IBRs around 15; **Figure 4.2.5.A**). In contrast, fish exposed to warming alone presented not only a lower IBR value in fish liver (i.e. IBR = 15) compared to the ones obtained in Acid, Acid+VFX-feed and Warm+VFX-feed treatments (i.e. IBRs = 18, 18 and 21, respectively), but also the highest IBR in the brain (i.e. IBR = 24; **Figure 4.2.5.A**). Interestingly, gills of non-contaminated fish exposed to acidification or warming showed higher IBR index values than those co-exposed to VFX via feed (i.e. 18.6 in Acid treatment against and 10.3 in Acid+VFX-feed treatment, and 13.7 in Warm treatment against and 9.8 in Warm+VFX-feed treatment; **Figure 4.2.5.A**). As for the combination of acidification plus warming, higher IBR index values were always obtained when VFX exposure was also added to the equation, i.e. Acid+Warm treatment always present lower IBR index values (mean IBR = 13) than Acid+Warm+VFX-water and Acid+Warm+VFX-feed treatments (**Figure 4.2.2.**). Moreover, Acid+Warm+VFX-water treatment also revealed higher values than Acid+Warm+VFX-feed treatment with the exception of fish muscle (i.e. 19), therefore yielding the highest mean IBR index out of all treatments (i.e. mean IBR all tissues combined = 21; **Figure 4.2.5.A**).

Concerning the contribution of each analysed biomarker to the total IBR index value, differential patterns were observed according to tissue and treatment (**Figures 4.2.5.B-Q** and **Annex 6, Table A.6.4.**). Starting with the CTR treatment, the most responsive biomarker was SOD (i.e. SOD scores within the four highest values in all tissues) followed, in this order, by CAT (in muscle, gills and brain), GST (in gills and liver) and LPO (in liver and brain). Conversely, lower scores were always attributed to HSP70/HSC70, VTG and AChE (**Figures 4.2.5.B-Q** and **Annex 6, Table A.6.4.**).

As for treatments simulating the exposure to stressors, SOD also consistently presented high scores, as did CAT in fish gills (except in Acid and Acid+VFX-feed, in which the IBR index value was overruled by GST instead) and LPO in the brain (**Figures 4.2.5.B-Q** and **Annex 6,**

Table A.6.4.). In general, Ub and VTG provided important contributions to the total IBR indexes in the muscle and liver of fish exposed to acidification and/or VFX via feed (i.e. VFX-feed, Acid and Acid+VFX-feed treatments; (**Figures 4.2.5.B-Q** and **Annex 6, Table A.6.4.**). In addition, these two biomarkers also played an important role in the liver of fish from Warm+VFX-feed and Acid+Warm+VFX-water treatments (**Figures 4.2.5.B-Q** and **Annex 6, Table A.6.4.**). On the other hand, fish muscle (except in Warm+VFX-feed and Acid+Warm+VFX-feed treatments) and liver (all treatments) exposed to warmer seawater temperatures usually exhibited high scores of HSP70/HSC70, regardless of pCO_2 levels (**Figures 4.2.5.B-Q** and **Annex 6, Table A.6.4.**). Finally, it is also worth mentioning that fish brain also denoted important contributions of AChE in Acid+Warm and Acid+Warm+VFX-water treatments, but not in Acid+Warm+VFX-feed treatment, in which higher scores were rather attributed to HSP70/HSC70 (**Figures 4.2.5.B-Q** and **Annex 6, Table A.6.4.**).

4. Discussion

4.1. Differential biomarker responses to VFX exposure route

Studies assessing the effects of exposure route on contaminants' uptake and toxicity to marine biota are still scarce and, so far, to the best of our knowledge, most studies on the ecotoxicological implications of antidepressants have only focused on water exposure (e.g. Brooks et al., 2014; Fong and Ford, 2014; Chen et al., 2018; Pan et al., 2018). Although PPCPs are, in general, assumed to be mostly uptaken from water by marine biota, our previous study with *A. regius* constituted a proof of concept that, indeed, dietary exposure can substantially contribute to the total contaminant body burden in fish, even if to a lower extent compared to water exposure, with fish liver being the primary organ for VFX bioaccumulation, regardless of exposure route (Maulvault et al., 2018a). Here, we provide relevant and innovative data that point out that: i) distinct tissue biochemical responses (i.e. no effects or up/down-regulations) are triggered when different pathways of antidepressants' exposure take place (i.e. water and diet); and ii) such differential tissue responses are not necessarily linked to the corresponding VFX tissue burdens elicited by the two VFX exposure routes (i.e. higher VFX tissue concentrations elicited by water exposure; Maulvault et al., 2018a); in fact, it seems that the exposure pathway influenced VFX toxicity, as well as tissue susceptibility to this compound.

Fish antioxidant mechanisms were altered by both VFX exposure routes, namely through the enhancement of CAT (in the liver) and GST (in the muscle) activities, a mechanism that is frequently activated to overcome the excessive formation of reactive oxygen species (ROS) induced by the exposure to stressors. On the other hand, muscle and gills showed diminished CAT activity (under VFX-feed exposure), as well as SOD activity (under both exposure routes). Such inhibition is likely associated with the fact that the antioxidant machinery was unable to

compensate for an excessive production of substrate (i.e. superoxide radicals are converted into H_2O_2 by SOD, and then CAT converts H_2O_2 into H_2O and O_2) induced by VFX exposure (Gonzalez-Rey and Bebianno, 2014; Maulvault et al., 2018c). GST activity inhibition in the liver of fish exposed to VFX via water, but not in those exposed via feed, might also be related with the higher VFX concentration elicited by VFX water exposure (i.e. ~6810 µg kg^{-1} in VFX-water treatment against ~150 µg kg^{-1} in VFX-feed treatment; values previously reported in Maulvault et al., 2018a), which could have exhausted liver's mechanisms of xenobiotic detoxification, through a decreased formation of reduced glutathione (i.e. lower substrate to be used by GST; Gonzalez-Rey and Bebianno, 2011). Furthermore, such impairment of VFX's detoxification in fish liver may justify the increased formation of lipid peroxides (i.e. increased MDA concentration) found in all fish tissues from VFX-water treatment. Hence, results suggest that tissue antioxidant defences were able to prevent to some extent the oxidative stress induced by VFX exposure via feed, but not by VFX exposure via water, promoting cell damage in fish subjected to this treatment. In accordance with our findings, previous studies on aquatic species exposed to antidepressants via water reported not only increased CAT and GST activities (in *Dreissena polymorpha*; Magni et al., 2016; *Danio rerio* and *Carassius auratus*; Pan et al., 2018), but also diminished SOD (in *Daphnia magna*; Ding et al., 2017) and GST (in the liver of *Pseudorasbora parva*; Chen et al., 2018) activities, both being accompanied by increased LPO (Ding et al., 2017; Chen et al., 2018). For instance, Chen et al. (2018) reported decreased CAT and GST activities alongside with increased LPO after long-term exposure to a high concentration of fluoxetine (200 µg L^{-1}). Yet, in this same study, a different pattern was observed under the lowest fluoxetine concentration (i.e. 50 µg L^{-1}), i.e. CAT activity increased after short-term exposure, but not in a long-term, while GST was not significantly affected (regardless of exposure duration; Chen et al., 2018). Hence, such concentration- and time-dependency in antidepressants mode of action may explain the differential effects elicited by the two pathways of VFX exposure in the present study.

The increased HSP70/HSC70 and Ub contents in fish exposed to VFX through both exposure routes is in agreement with the fact that the synthesis of these proteins is usually induced by the exposure to pollutants (e.g. Gravel and Vijayan, 2007; Horst et al., 2007; Ajima et al., 2018; Maulvault et al., 2018c), in order to prevent irreversible DNA damage, as the first mediates the repairing, refolding and elimination of damaged proteins (Sottile and Nadin, 2018), while the second is responsible for inactivating and tagging damaged proteins that are to be degraded by the proteasome (Jackson and Durocher, 2013). Yet, results suggest that VFX water exposure promoted cellular damage to a much higher extent compared to VFX feed exposure, given the substantial HSP70/HSC70 and Ub contents increase observed in fish gills, liver and brain from VFX-water treatment.

In opposition, VFX exposure via feed had a clear effect on AChE brain activity and VTG liver content (i.e. both increased), while no significant effects were elicited by VFX water exposure, despite the much higher VFX tissue concentrations observed in these tissues under VFX-water exposure (Maulvault et al., 2018a). The currently available literature has revealed some controversy in what concerns the effects of antidepressants on AChE activity in aquatic species, with some studies reporting its inhibition (e.g. Munari et al., 2014; Ding et al., 2017; Yang et al., 2018), and others describing an induction (e.g. Gonzalez-Rey and Bebianno, 2013; Rodrigues et al., 2014; Xie et al., 2015; Chen et al., 2018; Pan et al., 2018). Several authors have previously argued that antidepressants act on AChE in a time- (Gonzalez-Rey and Bebianno, 2013; Ding et al., 2017; Pan et al., 2018) and concentration-dependent (Munari et al., 2014; Rodrigues et al., 2014; Yang et al., 2018) manner, therefore, justifying the differential effects on AChE brain activity induced by the two VFX exposures routes simulated in our study. For instance, Munari et al. (2014) reported AChE activity inhibition in clams (*Venerupis philippinarum*) exposed to 1 and 5 µg L^{-1} of fluoxetine, but not in clams exposed to 25, 125 and 625 µg L^{-1}. Conversely, crab specimens *Carcinus maenas* exposed to sertraline-contaminated seawater exhibited an up-regulation of AChE activity (in muscle) under a low compound concentration (0.05 µg L^{-1}) and, concomitantly, a down-regulation under a high concentration (5 µg L^{-1}; Rodrigues et al., 2014). In the present study, the increased AChE activity elicited by VFX-feed exposure (acting alone) can have three potential justifications: i) VFX-feed exposure could have promoted brain cell apoptosis, causing the release of AChE from brain cells (Zhang et al., 2002; Gonzalez-Rey and Bebianno, 2013, 2014); ii) similar to what has been reported for human subjects, exposure to stressful conditions can increase the synthesis of AChE splicing variants (e.g. AChE-R; Lionetto et al., 2013); and iii) as previously reported for other antidepressants [e.g. increased VTG1 gene expression in brain and gonads of *Danio rerio* following the exposure to mianserin (van der Ven et al., 2006); inhibition VTG-like proteins (measured indirectly as alkali-labile phosphates measurement in *Mytillus galloprovincialis* exposed to fluoxetine (Gonzalez-Rey and Bebianno, 2014)], VFX (at the tissue and plasma concentrations elicited by feed exposure) might have had an estrogenic effect through the disturbance of the hypothalamo–pituitary–gonadal (HPG) axis, causing increased estrogen and VTG-like protein levels which, in turn, modulated the cholinergic system, including AChE activity (van der Ven et al., 2006; Gonzalez-Rey and Bebianno, 2014; Oliveira et al., 2015). The second and/or third hypothesises seem more plausible, since increased brain cell apoptosis would also presume increased LPO and altered chaperone and ubiquitin contents, which was not the case in VFX-feed treatment. Moreover, the third argument also matches the induction of VTG synthesis observed in the liver of VFX-feed exposed fish. Yet, further research on fish neuroendocrine responses to antidepressants (particularly, studies assessing AChE splicing forms, neurotransmitters, as well as sexual and thyroid hormone levels) are required in the future to confirm these hypothesises.

4.2. Interactive effects of warming and acidification

Overall, results evidenced that both warming and acidification strongly influenced fish coping mechanisms to the presence of antidepressants, resulting in either an enhancement of tissue ecotoxicological responses or in their attenuation/reversion. Such differential tissue responses are likely related to the fact that each tissue has distinct physiology, functioning and baseline levels of biochemical biomarkers, as they are composed by different cell types, and, therefore, may also respond differently to the interactive effects of environmental stressors.

Seawater warming and acidification have the potential to directly or indirectly influence marine species physiology, metabolism and overall fitness (Rosa et al., 2014, 2016, 2017; Madeira et al., 2016, 2018). Though many organisms have evolved to cope with daily or seasonal abiotic variations, their thresholds of physiological tolerance can be surpassed when the co-exposure to multiple stressors occurs at the same time (including exposure to pollutants), thus, compromising their fitness and ecological success (e.g. Rosa et al., 2016; Maulvault et al., 2017, 2018c; Sampaio et al., 2018). In this way, the additional physiological stress promoted by the co-exposure of VFX with abiotic stressors triggered cells antioxidant machinery (e.g. CAT in gills of fish exposed to VFX via water; CAT and SOD in brain of fish exposed to both VFX exposure routes, GST in muscle and brain of fish exposed to both VFX exposure routes). Although research on the interactive effects of climate change-related stressors is still in its infancy and, therefore, the available literature is limited, recent studies on marine biota have also reported a significant increase of CAT, SOD and GST activities when pollutants are co-exposed with higher temperatures and/or pCO_2 levels (e.g. Freitas et al., 2016; Maulvault et al., 2017, 2018c; Sampaio et al., 2018). Such enhancement occurs as a way to compensate the elevated formation of ROS, due to an intensive mitochondrial respiration along with diminished fish aerobic scope (and deprived oxygen supply to the different tissues; Heise et al., 2006; Pörtner and Peck, 2010). Nevertheless, cells' defences against low oxygen supplies and oxidative stress are time-dependent (Pörtner, 2002; Heise et al., 2006; Madeira et al., 2016, 2018), two different strategies may simultaneously take place under chronic stress conditions (such as the ones simulated in the present study, i.e. 28 days of exposure trial): i) after a certain period of acclimation, an organism may reach a state of internal homeostasis which enables to withstand stress and return to baseline levels (Madeira et al., 2018), i.e. not evidencing significant biomarker changes in relation to CTR, as occurred with CAT and SOD liver activities (all treatments, except Acid+Warm+VFX-water); or ii) when stress conditions are too severe and acclimation is no longer possible, animal metabolism can become depressed (and, consequently, protein synthesis is disturbed), translating into an inhibition of these enzymes' activities (Sokolova, 2013; Ferreira et al., 2015; Madeira et al., 2016; Maulvault et al., 2018c), e.g. as observed in CAT muscle activity (all treatments, except Acid+Warm+VFX-feed) and GST liver activity (in treatments simulating acidification, except

when the three stressors were combined). Moreover, an attenuation (or even reversion) of tissue responses could also be observed, in some cases, when the co-exposure to different stressors occurred (e.g. SOD activity in fish muscle was inhibited by increased temperature acting individually, but such inhibition was lowered by the co-exposure to acidification, whereas the co-exposure to VFX-feed did not induce significant alterations in relation to CTR treatment, regardless of pCO_2 levels). Similarly, in an earlier study using seabass (*Dicentrachus labrax*), diclofenac dietary exposure significantly decreased CAT activity (80% inhibition in relation to the control treatment), yet such inhibition was attenuated by the co-exposure to warming (63% inhibition) and/or acidification (57% inhibition; Maulvault et al., 2018c). The significant increase of LPO in all treatments (with the exception of VFX-feed) in relation to relation to CTR treatment indicated that, despite tissues' antioxidant defences were activated to some point (and extent), cell damage or even apoptosis occurred after 28 days of exposure to the studied stressors (particularly to acidification). Noteworthy, such tissue damage was particularly evident in fish gills (i.e. high concentrations of MDA in all treatments), and that could be related to the fact that gills are one of the most aerobic fish tissues (being responsible for fish breathing) and, therefore, are expected to be particularly sensitive to reduced oxygen levels caused by an impaired animal aerobic scope.

As previously mentioned, stressful environmental conditions can trigger the synthesis of molecular chaperones, such as HSP70/HSC70, as a way to repair reversible protein damage that antioxidant scavengers alone are not able to prevent (Madeira et al., 2017; Sottile and Nadin, 2018). Subsequently, when irreversible protein anomalies occur (i.e. molecular chaperoning mechanisms no longer can repair the cellular damage), the ubiquitin-proteosomal pathway is also initiated to signal and eliminate such proteins (Jackson and Durocher, 2013; Madeira et al., 2017). Yet, since protein synthesis is an extremely demanding process from the energetic point of view (requiring over 50% of an organism's total oxygen supply), both mechanisms of chaperoning and ubiquitination can be impaired when organisms fall into physiological collapse due to severe or long-lasting stress conditions (Hofmann and Somero, 1995; Gravel and Vijayan, 2007; Araújo et al., 2018; Maulvault et al., 2018c). Based on this background knowledge, the present results suggest that, overall, increased temperatures (i.e. Warm, Warm+VFX-feed, Acid+Warm, Acid+Warm+VFX-feed and Acid+Warm+VFX-water treatments) promoted reversible cellular damaged in fish brain, which was withstood by the induction of HSP70/HSC70. Although the diminished HSP70/HSC70 and unchanged Ub contents in fish liver from Warm+VFX-feed and Acid+Warm+VFX-feed treatments could indicate, at a first glance, impaired cellular responses (Araújo et al., 2018), the trends of antioxidant enzymes and LPO in these treatments indicate that the physiological state of fish liver was favoured by warming, possibly due to an enhanced animal metabolism (and, thus, enzymatic activity and protein synthesis) which could have counteracted the negative effects of VFX feed exposure and/or acidification. In contrast, the simultaneously co-exposure to VFX via water, acidification and warming seemed to have caused severe

impairments in tissues' protective mechanisms, as revealed by the increased chaperone synthesis in fish muscle, gills and brain, together with the inhibition of these proteins in the liver and of Ub in the muscle. Such depressed physiological stated could be likely attributed to the higher VFX tissue burdens elicited by VFX water exposure (in relation to VFX feed exposure, regardless of abiotic stressors; Maulvault et al., 2018a), which deteriorated fish aerobic scope and impaired protein synthesis (Falfushynska et al., 2014; Madeira et al., 2017).

Seawater abiotic conditions, such as temperature and pH, play a key role on fish reproduction, determining the success of oocyte maturation, ovulation and spawning (e.g. Brown et al., 2006; Arantes et al., 2011; Milazzo et al., 2016). In line with this, both climate change-related stressors affected *A. regius* neuroendocrine response regardless of VFX exposure, with warming being responsible for an induction of VTG production and AChE activity, whereas acidification not only inhibited VTG synthesis but also increased AChE activity, though to lower extent compared to warming. This overstimulation of fish neuroendocrine responses in treatments simulating warming (regardless of VFX exposure) is in accordance with previous findings on the effects of temperature and chemical pollutants (endocrine disrupting compounds; Chandra et al., 2012; Maulvault et al., 2018c; Shappell et al., 2018). For instance, Chandra et al. (2012) reported increased VTG1 mRNA gene expression in *Fundulus heteroclitus* male specimens exposed to the combination of 17α-ethynylestradiol and increased seawater temperature (26 ºC). As hypothesized by these authors, such enhancement is certainly related to the exacerbation of fish metabolic rates (and, consequently, enzyme activities) at warmer temperatures. Yet, it should be noted that drastic temperature variations (i.e. outside species' physiological thresholds) have also been associated with inhibitory reproductive effects in teleost species (Pankhurst and Munday, 2011; Miranda et al., 2013). Therefore, the present results suggest that, despite the elicited changes, a temperature increase of +5 ºC may still fall within the reproductive thermal window of *A. regius*. The present results are also consistent with the trends observed in our previous studies with *D. labrax* (Maulvault et al., 2018c) and *Diplodus sargus* (Maulvault et al., 2018d), as increased pCO_2 levels evidenced an anti-estrogenic effect (i.e. VTG inhibition) and cholinergic modulation, possibly due to a disturbance of brain ionic homeostasis which, in turn, impaired neurotransmission and hormone synthesis (Pankhurst and Munday, 2011; Nilsson et al., 2012; Kwong et al., 2014; Heuer et al., 2016). Yet, such effects seemed to have been attenuated by warming and, to a lower extent, by VFX feed exposure, but not by VFX water exposure, once again, pointing out differential tissue responses to VFX exposure route. Since, so far, research on the reproductive effects of acidification (as well as warming) has been mostly focused on sensory and behavioural aspects (e.g. Nilsson et al., 2012; Munday et al., 2014; Maulvault et al., 2018b), further studies in this direction are still required to better understand the biochemical processes involved in fish neuroendocrine responses to climate change-related stressors.

4.3. Using IBRs to estimate the overall fish fitness

To fully understand the effects of environmental stressors can become a challenging task, especially, when different tissues with distinct sensitivity and biomarker responses are analysed, as well as when multiple stressors interact with each other. Hence, IBRs constitute an innovative and practical tool that enables a qualitative assessment of the overall fitness of organisms, as well as comparisons among different stressors according to their magnitude of severity (e.g. Kamel et al., 2014; Ferreira et al., 2015; Madeira et al., 2016, 2018; Maulvault et al., 2018c). The use of this tool also evidences the sensitivity of each biomarker and tissue to respond to a specific stressor (Madeira et al., 2016, 2018; Maulvault et al., 2018c).

Starting with biomarkers' sensitivity to the studied stressors, the fact that SOD and LPO consistently yielded high scores, regardless of tissue and treatment, pointed out to a lack of specificity when responding to the three studied stressors acting individually or to their interactions. Conversely, in accordance with previous findings (Madeira et al., 2016, 2018), HSP70/HSC70 content proved to be a sensitive biomarker of thermal stress (in fish muscle, liver and brain), despite its induction was reduced or even inhibited by the co-exposure to VFX or acidification. Even though no previous IBR studies specifically focusing on the effects of acidification and antidepressants exposure were found, the present data showed an overall good reactivity of Ub and liver VTG content to acidification, thus, confirming the cytotoxic and anti-estrogenic potential of increased pCO_2 levels (Maulvault et al., 2018c).

Using IBRs to compare stressors severity, as far as the effect of VFX exposure route is concerned, results evidenced that fish muscle, gills and liver were more susceptible to VFX exposure via feed (i.e. higher IBR) than to VFX water exposure, and such susceptibility was mostly attributed to the remarkable changes induced by this exposure pathway in CAT activity, as well as in Ub and VTG contents. Yet, the remarkable increase in LPO, protein chaperoning and degradation in fish exposed to VFX via water resulted in a much higher degree of stress in fish brain (i.e. higher IBR). The substantial increase of these biomarkers' scores also translated into a poorer physiological state of water exposed fish (i.e. higher average IBR index, combining the integrated responses of all tissues) compared to those exposed via feed, a result that is in line with the differential VFX tissue concentrations elicited by both exposure routes (values previously reported in Maulvault et al., 2018a).

Regarding the interactive effects of climate change-related stressors, regardless of the remarkable effects on Ub and VTG liver contents, IBR results revealed that acidification had an overall negative impact on fish gills (promoting severe cell damage, according to the increased LPO and Ub contents), but its effects were less evident in the remaining tissues compared to those promoted by warming and/or VFX exposure. In contrast, warming alone proved to be particularly harmful to fish brain. This can be attributed to the enhanced tissue metabolic rates (i.e. increased

CAT, GST and AChE activities and HSP70/HSC70 content) together with increased LPO. The comparatively lower IBR in fish brain from Warm+VFX-feed treatment was mainly due to the fact that VFX feed exposure attenuated biomarker changes induced by warming. The co-exposure to the three stressors (i.e. Acid+Warm+VFX-feed and Acid+Warm+VFX-water treatments) resulted, overall, in higher IBR index values, regardless of the lower VFX tissue concentrations elicited by these treatments (values presented in Maulvault et al., 2018a). These results reveal that, indeed, temperature and pCO_2 levels have a determinant role in fish fitness, especially when both abiotic stressors are combined. In line with these findings, the co-exposure to pollutants and abiotic stressors has been previously described to result in comparatively higher IBR values, leading to a poorer animal physiological state (Serafim et al., 2012; Kamel et al., 2014; Ács et al., 2016; Maulvault et al., 2018c). As occurred under normal temperature and pCO_2 conditions, IBR data also highlighted that a higher magnitude of stress was inferred when VFX exposure occurred via water (i.e. higher tissue IBR in Acid+Warm+VFX-water treatment compared to Acid+Warm+VFX-feed treatment, except in fish muscle).

As a final remark, it is also worth noting that the good responsiveness of AChE brain activity in fish co-exposed to the three stressors matched *A. regius* decreased exploratory behaviour and shoal cohesion observed in our earlier study (Maulvault et al., 2018b), pointing out severe neurological impairments, most likely linked to disrupted neurotransmission and/or brain cells' death.

5. Conclusions

In this study, we show in a comprehensive way that VFX toxicological attributes to marine fish species are strongly influenced by the uptake pathway, as well as by the surrounding abiotic conditions. Furthermore, our results highlighted the importance of analyzing multiple tissue responses as to have a broader view of fish ecotoxicological responses, since each tissue is structurally and functionally distinct and, therefore, can respond differently to the presence of environmental stressors. As evidenced by our data, the differential tissue responses to stressors can translate into either an enhancement of biomarker levels (e.g. increase in CAT activity, LPO, Ub and VTG liver contents due to VFX exposure) or an inhibition (e.g. decreased CAT and SOD activities in muscle and gills, respectively, due to VFX exposure, warming or acidification). In addition, when multiple stressors interact with each other, such effects can be either exacerbated (e.g. CAT activity in fish muscle further decreased by the combination of VFX with acidification or warming) or attenuated/counteracted (GST activity in the liver was inhibited by acidification or warming, but such inhibition was attenuated by VFX feed exposure).

By integrating all tissue biomarker responses, it became evident that the physiological stress induced by VFX water exposure was more severe (i.e. higher mean IBR index value) compared

to VFX feed exposure, regardless of seawater temperature and pCO_2 levels. As for the interactive effects of abiotic stressors, while warming was generally associated to a poorer fish physiological state, the negative impact of acidification was only clearly evident in fish gills. Finally, the combination of the three stressors corresponded to the most severe stress scenarios (particularly, following VFX water exposure), overall yielding higher IBR index values than treatments simulating stressors acting alone or the interaction of two stressors. Hence, the present results emphasize the importance of conducting multi-stressor ecotoxicological assessments to enable a deeper understanding of the consequences of climate change, as well as to develop region-specific mitigation strategies, since environmental stressors will rarely occur in isolation and their ecological impacts will not be felt in the same way across the planet.

Ethical statement

Fish trials were carried out following the EU ethical requirements (EU Directive 2010/63), being approved by the Ethical Committee of the Faculty of Sciences of the Lisbon University, overseen by the Portuguese National Competent Authority (Direção-Geral de Alimentação e Veterinária, DGAV). All researchers involved in fish trials were previously accredited by the Federation of European Laboratory Animal Science Associations (FELASA).

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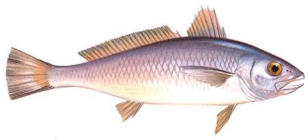
Part 3.

Differential behavioural responses to venlafaxine exposure route, warming and acidification in juvenile fish (*Argyrosomus regius*)

Manuscript 6.

Maulvault, A.L., Santos, L.H.M.L.M. Santos, Paula, J.R., Camacho, C., Pissarra, V., Fogaça, F., Barbosa, V., Alves, R., Pousão Ferreira, P., Barceló, D., Rodriguez-Mozaz, S., Marques, A., Diniz, M., Rosa, R., 2018. Differential behavioural responses to venlafaxine exposure route, warming and acidification in juvenile fish (*Argyrosomus regius*). *Science of the Total Environment*, 634, 1136–1147. DOI: 10.1016/j.scitotenv.2018.04.015.

Graphical abstract



Argyrosomus regius
juveniles

28 days of exposure to three stressors:

- Venlafaxine (water: 20 µg L^{-1}; feed: 160 µg kg^{-1})
- Seawater warming (+ 5° C)
- Seawater acidification (+ 1000 µatm pCO_2)

VFX:
- Increased exploration
- Decreased activity and shoal cohesion

Acidification + VFX:
- Decreased exploration and shoal cohesion
- Reversed side preference

Warming + Acidification + VFX:
- Increased VFX plasma concentration
- Decreased shoal cohesion
- Loss of lateralization

Need to consider the likely interactive effects of seawater warming and acidification in future research regarding the toxicological aspects of chemical contaminants

Abstract

Antidepressants, such as venlafaxine (VFX), which are considered emerging environmental pollutants, are increasingly more present in the marine environment, and recent evidence suggest that they might have adverse effects on fish behaviour. Furthermore, altered environmental conditions associated to climate change (e.g. warming and acidification) can also have a determinant role on fish behaviour, fitness and survival. Yet, the underlying interactions between these environmental stressors (pharmaceuticals exposure and climate change) are still far from being fully understood. The aim of this study was to assess behavioural responses (in juvenile meagre (*Argyrosomus regius*) exposed to VFX via water ([VFX] ~20 µg L^{-1}) and via dietary sources ([VFX] ~160 µg kg^{-1} dry weight), as well as to increased temperature ($\Delta T^{o}C$ = +5 ºC) and high CO_2 levels (Δ pCO_2 ~1000 µatm; equivalent to ΔpH = -0.4 units). Overall, VFX bioaccumulation in fish plasma was enhanced under the combination of warming and acidification. VFX triggered fish exploration, whereas fish activity and shoal cohesion were reduced. Acidification alone decreased fish exploration and shoal cohesion, and reversed fish preference to turn leftwards compared to control conditions. Such alterations were further enhanced by VFX exposure. The combination of warming and acidification also reduced shoal cohesion and loss of lateralization, regardless of VFX exposure. The distinct behaviour observed when VFX contamination, acidification and warming acted alone or in combination highlighted the need to consider the likely interactive effects of seawater warming and acidification in future research regarding the toxicological aspects of chemical contaminants.

Keywords: Fish behaviour, antidepressants, venlafaxine, ocean warming, ocean acidification.

1. Introduction

Pharmaceuticals and personal care products (PPCPs) have become a great environmental concern, since they are a group of compounds intensively and continuously used, and their presence in both the environment and biota is currently not regulated. Domestic, hospital and industrial effluents, agriculture and aquaculture activities are the main sources of PPCPs' contamination in marine environments (Gros et al., 2012), and their elimination through conventional wastewater treatments is chemically dependent and only partial (e.g. 50% of maximum removal for venlafaxine, lidocaine and tramadol; Rúa-Gómez and Püttmann, 2012). Given their ability to easily cross biological membranes, and high specificity and effectiveness to target cells and tissues, PPCPs can be toxic to non-target organisms, even at very low concentrations (e.g. Mehinto et al., 2010; Schmidt et al., 2011). Such impacts may be more deleterious when long-term or chronic exposure occurs, particularly in early life stages that are known to possess lower capabilities to metabolize such contaminants (Richardson and Ternes, 2011). Nevertheless, the ecological impacts of PPCP exposure still require a better understanding, as most available studies do not consider chronic (but sub-lethal) exposures (Dulawa et al., 2004), and are focused on bioconcentration rates (i.e. contaminants exposure via water), while other exposure routes, such as trophic transfer (i.e. dietary exposure) have deserved very little attention, despite they can be particularly important in predatory fish species that have long life cycles and are able to reach relatively high body dimensions (Dijstra et al., 2013; Brooks et al., 2014; Zenker et al., 2014; Maulvault et al., 2016). Furthermore, very few studies consider the effect of other stressors, such as climate change (e.g. warming, acidification), which can affect PPCPs' bioavailability (Brooks, 2014).

Within pharmaceuticals of human use, venlafaxine (VFX) is frequently detected in the aquatic environment (e.g. around 50 ng L^{-1} in seawater samples and up to 580 ng L^{-1} in wastewater influent; Gos et al., 2012), often reaching higher concentrations than other well-known psychiatric drugs, such as fluoxetine or carbamazepine (Gros et al. 2012; Fong and Ford, 2014). VFX acts as a behaviour modulator by blocking the presynaptic reuptake of serotonin and norepinephrine (Serotonin-norepinephrine reuptake inhibitor; SNRI). This results in increased serotonin and norephinephrine in the synapse, which then is available to bind to postsynaptic receptors and cause increased downstream effects *(*Thaler et al., 2012*)*. From the evolutionary perspective, vertebrate species have many preserved neurotransmitter systems and receptors, which is why many antidepressants that act on humans have similar effects on fish (e.g. Valenti et al., 2012; Bisesi Jr. et al., 2014). However, studies on antidepressants and fish are limited and detailed toxicological information is required to better understand the effects of these compounds as wastewater pollutants (Brodin, et al., 2013; Hamilton et al., 2017). Empirical data establishing toxicological and behavioural similarities (or distinctions) between humans, primates and other

vertebrate organisms, such as fish, exposed to antidepressants are important in two ways: i) to investigate fish species' potential as *in vivo* experimental models that complement the data provided by mammalian models in neurotoxicological studies, since laboratory studies using humans and primates are often difficult, time consuming, costly and underlie many ethical issues; and ii) to assess possible ecological implications and cascading effects to marine biota due to the environmental contamination related to human pharmaceuticals. Over the last decades, different tests have been developed and validated to assess distinct behavioural cues in fish species, such as animal anxiety (e.g. novel tank diving test; Bencan et al., 2009; Sackerman et al., 2010; Reyhanian et al., 2011; Stewart et al., 2012) and social interactions (e.g. the shoaling test; Moretz et al., 2007; Reyhanian et al., 2011). Moreover, during the last two decades, fish lateralization has been one of the main research areas in fish behavioural studies (e.g. Bisazza and Brown, 2011; Bibost and Brown, 2013; Sampaio et al., 2016), because: i) it is intrinsically involved in habitat exploration, synchronized and polarized group swimming (schooling), as well as in fish loose group aggregation (shoaling), thus contributing to enhance foraging and predator escape (e.g. Bisazza and Dadda, 2005; Bibost and Brown, 2013); ii) recent evidence suggest that lateralization is an ecological strategy required to meet the contemporary ecological and social demands involved in the processes of natural selection (e.g. Bisazza and Dadda, 2005; Bisazza et al., 2000; Bisazza and Brown, 2011; Bibost and Brown, 2013).

One third of the anthropogenically-originated carbon dioxide (CO_2) has been absorbed by the oceans, which has led to a 0.1 unit drop in seawater pH from the pre-industrial to the present days (IPCC, 2014). Carbon dioxide concentrations have risen to concentrations now exceeding 400 ppm (NOAA, 2017), and are expected to reach ~900 ppm by the end of the 21st century (Pörtner et al. 2014). These consequent changes in seawater chemistry are underpinned by a net increase of hydrogen (H^+) and bicarbonate (HCO_3^-) ions and decrease in carbonate ions (CO_3^{2-}), a process known as ocean acidification (Caldeira and Wickett, 2004). By 2100, in a "business-as-usual" scenario, the continuous CO_2 uptake is expected to elicit a further 0.13-0.42 pH drop (IPCC 2014). Concomitantly, excessive greenhouse gas emissions (which are responsible for heat absorption and reemission) are also expected to promote a surface seawater temperature increase as high as +4.8 ºC (IPCC, 2014).Given the susceptibility of marine organisms to environmental variations, which can affect their physiological status and behaviour (e.g. Anacleto et al., 2014; Sampaio et al., 2016; Rosa et al. 2017), warming and acidification are two of the main challenges that species will have to face in a changing ocean (IPCC, 2014). By interfering with seawater physical and chemical properties, climate change can also affect the availability of chemical contaminants in marine ecosystems, their transfer among environmental compartments and their toxicity to biota (Marques et al., 2010; IPCC, 2014). Yet, it is still unclear how species will cope with the presence of chemical contaminants in climate change scenarios. Since behaviour plays a major role in an

organism's ecological fitness and survival, potential changes induced by chemical contaminants and climate change, as well as the combination of both stressors may lead to substantial consequences at populational and ecosystem levels.

Within this context, the present study aimed to assess VFX bioaccumulation (fish plasma) and the respective behavioural responses (anxiety, swimming activity, shoaling and lateralization) in juvenile meagre (*Argyrosomus regius*), when accounting for the effects of: a) VFX exposure route (via water, i.e. [VFX] ~20 µg L^{-1}, and via dietary sources, i.e. [VFX] ~160 µg kg^{-1}, dw); b) abiotic stressors, namely warming (ΔTºC = +5 ºC) and acidification (Δ pCO_2 ~1000 µatm; ΔpH = -0.4 units). Juvenile *A. regius* was selected as biological model because it is a predatory fish species that typically inhabits estuaries and coastal areas, thus being susceptible to accumulate high levels of chemical contaminants (FAO, 2017). Furthermore, the fact that it is also a commercially valuable species also emphasizes the relevance of using this species in ecotoxicological and behavioural studies, as changes to the behavioural patterns can potentially affect juvenile recruitment and species ecological success, therefore, affecting both fisheries and aquaculture sectors in an adverse way.

2. Materials and Methods

2.1. Feeds (CTR and VFX-enriched) and VFX stock solutions

Non-contaminated feed (control, CTR feed) and VFX contaminated feed (VFX-enriched feed) with the same nutritional composition were manufactured by the company SPAROS Lda (Olhão, Portugal). Detailed feed composition can be consulted in **Annex 7, Table A.7.1**. Briefly, a control diet (CTR feed) was formulated to mimic a commercial fishmeal-rich formulation for juvenile marine fish with 48% crude protein and 18% crude fat. All powder ingredients were grinded (< 200 micron) in a micropulverizer hammer mill (Hosokawa Micron, SH1, The Netherlands). Ingredients and fish oil were then mixed accordingly to the target formulation in a paddle mixer (Mainca RM90, Spain), and the feed mixture was further humidified with 25% deionized water at room temperature. The diet was extruded at 2.0 mm by means of a low-shear extruder (P55, Italplast, Italy). Upon extrusion, the feed pellets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). A 10 kg batch of CTR feed was subsequently contaminated with VFX (VFX-enriched feed). Given the current lack of background information, and to assure that behavioural changes were elicited during the timeline of the trials, a VFX nominal concentration of approximately 160 µg kg^{-1} on a dry weight basis (dw) was selected, which corresponds to ~4 times the values commonly found in species inhabiting contaminated coastal areas, susceptible to accumulate this contaminant, and that are natural preys of juvenile meagre (Álvarez-Muñoz et al., 2015). To prepare the VFX-enriched feed, venlafaxine hydrochloride ($C_{17}H_{27}NO_2 \cdot HCl$, >98%, CAS Number 99300-78-4, Sigma-Aldrich) previously solubilized in

ethanol, was further diluted in deionized water (total volume of 100 mL), and this solution was top-coated to the pellets with a pressurized spraying container (standard flat-fan nozzle; size 250 micron; pressure 6 bar). Despite the top-coating process followed leads to the total volatilization of organic solvents (and, therefore, ethanol is not expected to be present in the experimental feeds), equivalent amounts of ethanol were also added to the Control feed to rule out the possibility of occurring any solvent carrier toxicity through feed.

To perform VFX exposure via water (i.e. in VFX-water treatment), a stock solution of VFX was prepared to daily spike seawater during the 28 days of exposure, by dissolving venlafaxine hydrochloride ($C_{17}H_{27}NO_2 \cdot HCl$, >98%, CAS Number 99300-78-4, Sigma-Aldrich) with deionized water (total volume of 500 mL), in order to achieve a nominal VFX concentration of 20 µg L^{-1} in each incubating tank. Such VFX nominal concentration was mostly based on the order of magnitude of the lowest VFX concentration previously reported to cause significant behavioural effects in fish following short-term VFX exposure (50 µg L^{-1}; Bisesi Jr et al., 2014).

2.2. Fish rearing and acclimation

A. regius specimens (*n* = 135) with similar biometric characteristics were reared until juvenile stage (total length: 6.8 ± 0.6 cm; weight 2.6 ± 0.8 g; **Table 4.3.1.**) at the aquaculture pilot station of the Portuguese Institute for the Sea and Atmosphere (EPPO-IPMA, Olhão, Portugal) using routine hatchery conditions. Subsequently, fish were transported to the aquatic facilities of Laboratório Maritimo da Guia (MARE-FCUL, Cascais, Portugal), where they were randomly and equitably distributed in 27 rectangular shaped incubating glass tanks (3 replicates x 9 treatments = 27 tanks in total; treatments randomly assigned to each tank/replicate; **Figure 4.3.1.**; see the description of each treatment in **sections 2.3.1**. and **2.3.2**.), within independent recirculation aquaculture systems (RAS), each having 50 L of total volume capacity. Each of the 27 tanks had independent functioning, being equipped with protein skimmer (Reef SkimPro, TMC Iberia, Portugal), UV disinfection (Vecton 300, TMC Iberia, Portugal), biological filtration (model FSBF 1500, TMC Iberia, Portugal) and chemical filtration (activated carbon, Fernando Ribeiro Lda, Portugal) to maintain seawater quality. Furthermore, each tank had independent temperature and pH control, and these parameters were adjusted whenever needed by means of: i) temperature - an automatic seawater refrigeration system (± 0.1 °C; Frimar, Fernando Ribeiro Lda, Portugal), as well as submerged digital thermostats (200W, V2Therm, TMCIberia, Portugal); ii) pH - individual pH probes (GHL, Germany) connected to a computerized pH control system (± 0.1 pH units; scale: pH 0.0-14.0 units; Profilux 3.1N, GHL, Germany), which monitored seawater pH in each tank every 2 s, and adjusted whenever need, via submerged air stones, by injecting CO_2 (Air Liquide, Portugal; to decrease pH) or by CO_2-filtered aeration (to increase pH) using air pumps (Stella 200, Aqua One Pro, Aqua Pacific UK Ltd, United Kingdom).

Table 4.3.1. Fish weight (g), total length (cm), VFX concentrations and net accumulation rates (NAR) in plasma of specimens collected in each treatment (trials I and II; day 28. In each column, different letters indicate significant differences between treatments ($p < 0.05$). Abbreviations: LOD: method's limit of detection; nd – not determined; Acid – simulated acidification (i.e. pCO_2 ~1500 µatm, equivalent to pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC); VFX-water – fish exposed to VFX via water; VFX-feed – fish exposed to VFX via feed.

	Weight (g)	Total length (cm)	Plasma VFX concentration (µg L^{-1})	NAR (µg L^{-1} day^{-1})
Day 0 (all)	2.6 ± 0.8^{ab}	6.8 ± 0.6^{abc}	nd	-
Control	2.3 ± 0.7^{b}	6.1 ± 0.7^{bc}	< LOD	-
VFX-water	2.2 ± 0.5^{b}	6.3 ± 0.4^{bc}	1292.0 ± 79.9^{a}	45.88 ± 1.72^{a}
VFX-feed	2.2 ± 0.5^{b}	6.0 ± 0.6^{c}	13.5 ± 1.4^{c}	0.48 ± 0.03^{d}
Acid	2.9 ± 0.8^{ab}	6.9 ± 0.6^{abc}	nd	-
Acid+VFX-feed	2.4 ± 0.4^{b}	6.5 ± 0.4^{bc}	24.8 ± 8.5^{bc}	0.91 ± 0.18^{c}
Warm	4.9 ± 1.1^{a}	7.8 ± 0.8^{a}	nd	-
Warm+VFX-feed	4.7 ± 1.6^{a}	7.8 ± 0.8^{a}	34.9 ± 20.6^{bc}	1.18 ± 0.44^{bc}
Acid+Warm	4.8 ± 1.5^{a}	7.9 ± 0.5^{a}	nd	-
Acid+Warm+VFX-feed	5.0 ± 1.8^{a}	8.0 ± 1.2^{ab}	40.6 ± 11.7^{b}	1.41 ± 0.25^{b}

Dead fish and faeces were daily removed and 25 % seawater renewal was performed in each incubation tank. Ammonia, nitrite and nitrate levels were daily checked, by means of colorimetric tests (Tropic Marin, USA), and kept below detectable levels, with the exception of nitrates, which were kept below 2.0 mg L^{-1}. Fish density was kept below 1 g body weight L^{-1} (i.e. 5 fish in each 50 L replicate tank) in order to avoid physiological stress related to high animal density. Specimens were initially acclimated to laboratory conditions for 30 days, being fed with CTR feed (2% of average body weight, bw) and kept under the following abiotic conditions: i) dissolved oxygen (DO) > 5 mg L^{-1}; ii) temperature (T ºC) = 19.0 ± 0.5 °C; iii) pH = 8.00 ± 0.10 units; iv) salinity = 35 ± 1 ‰; and v) photoperiod = 12L:12D (12 hours light:12 hours dark). Temperature, pH, salinity and DO were daily checked using a multi-parameter measuring instrument (Multi 3420 SET G, WTW, Germany). Seawater total alkalinity was also measured in every tank on a weekly basis, following the protocol previously described elsewhere (Sarazin et al., 1999) and the combination of total alkalinity (AT) and pH was used to calculate carbonate system parameters (average values obtained for each treatment can be consulted in **Annex 7, Table A.7.2**).

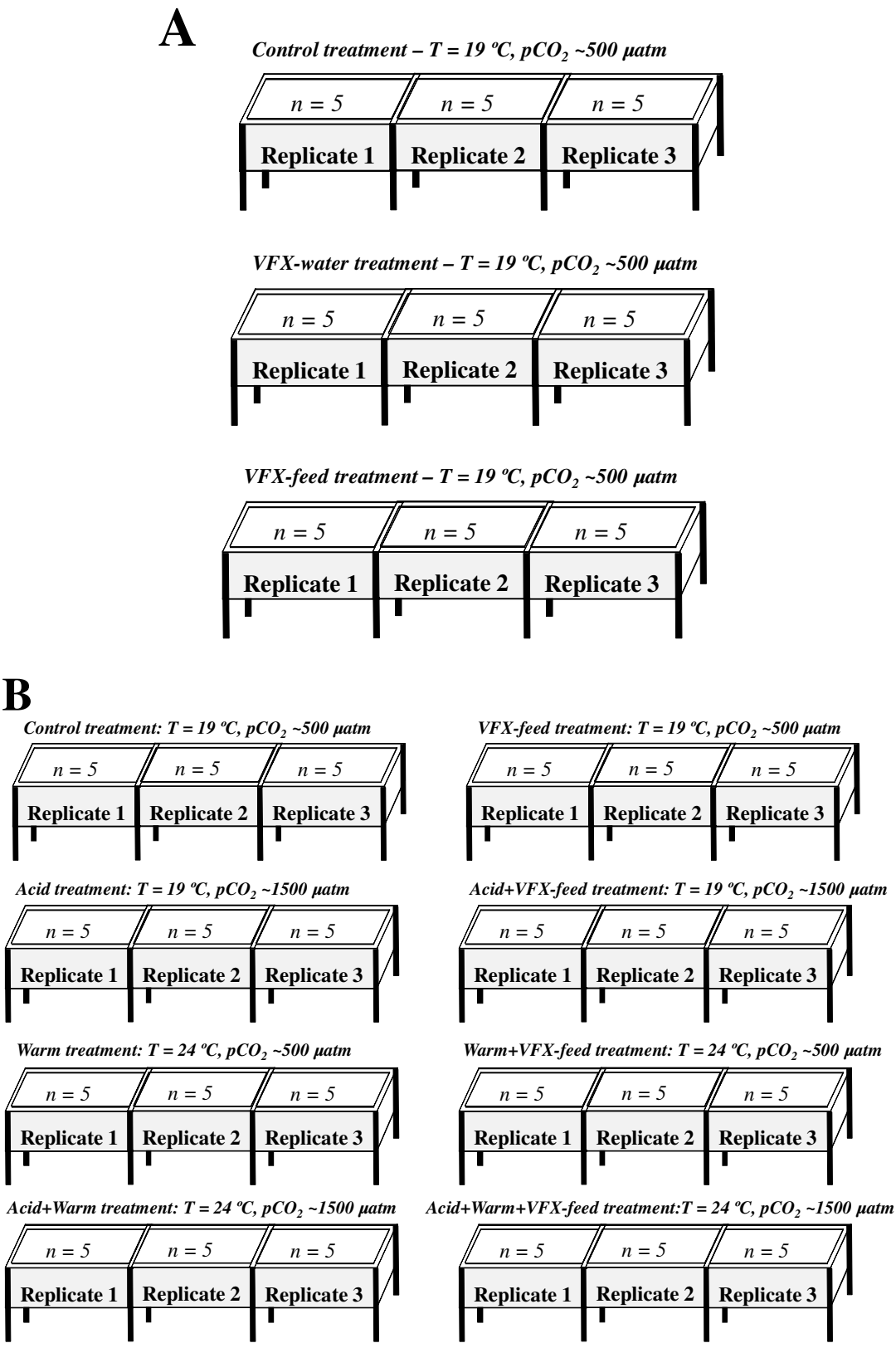


Figure 4.3.1. Experimental design of trials I - Seawater *versus* dietary exposure (A); and II - Simulation of climate change effects and VFX exposure via enriched feed (B). Abbreviations: Acid – simulated acidification (i.e. pCO_2 ~1500 µatm, equivalent to pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC); VFX-water – fish exposed to VFX via water; VFX-feed – fish exposed to VFX via feed.

2.3. Exposure to VFX

2.3.1. Trial I: Seawater versus dietary exposure

Three treatments were carried out (n = 5 animals per replicate tank, i.e. 15 animals per treatment; **Figure 4.3.1.A**), simulating the average seawater temperature and pH currently used in juvenile meagre rearing in the South Europe, i.e. reference temperature and pH conditions = 19 °C and 8.0 pH units: i) Control treatment, i.e. fish daily fed with CTR feed (2% bw); ii) VFX-water treatment, i.e. fish daily fed (2% bw) with CTR feed, and seawater daily spiked with a VFX stock solution (nominal concentration = 20 µg L^{-1} in the tank);; iii) VFX-feed treatment, i.e. daily fish fed (2% bw) with VFX-enriched feed (nominal concentration = 160 µg kg^{-1} dw). Seawater abiotic parameters were daily checked and adjusted to adequate levels whenever needed, as described above. No mortality was observed during the 28 days of trial. By the end of exposure, behavioural tests were conducted (see **section 2.5**) in ten animals randomly selected out of the three replicate tanks composing each treatment. Afterwards, fish were removed from the test tanks and euthanized by immersion in an overdosed MS222 solution (2000 mg L^{-1}; Sigma-Aldrich, USA) buffered with sodium bicarbonate (1 g of $NaHCO_3$ to 1 g of MS222 to 1 L of seawater) for 10 min. Euthanized fish were measured (total length and weight; **Table 4.3.1.**), and blood was collected by puncture of the caudal vein and centrifuged (4 ºC, 15 min, 10,000 *g*). Plasma samples were collected from the 10 fish for each treatment (3 tanks per treatment), pooled in two composite samples (n = 2) and kept at -80 ºC until further analyses.

2.3.2. Trial II: Simulation of warming and acidification

Due to experimental limitations, only one exposure pathway was selected to investigate the link between VFX exposure and climate change. Thus, exposure via VFX-enriched feed was selected for this purpose, because: a) contaminant exposure through dietary sources (i.e. trophic transfer of contaminants) currently represents a research gap in ecotoxicological studies; b) dietary exposure is thought to significantly contribute to high contaminant bioaccumulation in animal tissues, sometimes leading to more notorious toxicological effects than those promoted by contaminant exposure through inhalation, depending on the chemical behaviour of the target contaminant (e.g. Arnot and Gobas, 2004; Brooks et al., 2014).

One week before initiating VFX exposure, seawater temperature and pCO_2 were slowly adjusted (+1 ºC and -0.1 pH unit per day), until reaching 24 ºC and ~1500 µatm pCO_2 (equivalent to pH = 7.6 units) in tanks simulating climate change conditions (i.e. treatments Acid, Warm, Acid+Warm, Acid+VFX-feed, Warm+VFX-feed and Acid+Warm+VFX-feed; **Figure 4.3.1.B**; see also **section 2.3.**), according to the projections of the Intergovernmental Panel for Climate Change (scenario RCP8.5; IPCC, 2014). It is worth noting that the high pCO_2 levels used here (~1500 µatm) are beyond the worst-case IPCC scenarios for the end of the century (RCP8.5)

(IPCC, 2014), but still within the intervals of future CO_2 amplification scenarios described by McNeil and Sasse (2016).

Eight treatments were carried out (n = 5 animals per replicate tank of treatment, i.e. a total of 15 animals per treatment; **Figure 4.3.1.B**), simulating the reference temperature (i.e. 19 ºC) and pCO_2 (~500 µatm; 8.0 pH units) conditions, as well as the projected seawater warming (ΔTºC = +5 ºC) and acidification (ΔpCO_2 ~1000 µatm; equivalent to ΔpH = -0.4 units), using a full cross-factorial design: i) Control treatment, i.e. fish daily fed with CTR feed (2% bw) and exposed to reference temperature and pH conditions; ii) Acid treatment, i.e. fish daily fed with CTR feed (2% bw) and exposed to acidification (1500 µatm pCO_2, equivalent to pH = 7.6 units); iii) Warm treatment, i.e. fish daily fed with CTR feed (2% bw) and exposed to warming (24 °C); iv) Acid+Warm, i.e. fish daily fed with CTR feed (2% bw) and exposed to warming and acidification (24ºC and ~1500 µatm pCO_2); v) VFX-feed treatment, i.e. fish daily fed with VFX-enriched feed (2% bw) and exposed to reference temperature and pH conditions; vi) Acid+VFX-feed treatment, i.e. fish daily fed with VFX-enriched feed (2% bw) and exposed to acidification (1500 µatm pCO_2); vii) Warm+VFX-feed treatment, i.e. fish daily fed with VFX-enriched feed (2% bw) and exposed to warming (24 ºC); viii) Acid+Warm+VFX-feed treatment, i.e. fish daily fed with VFX-enriched feed (2% bw) and exposed to acidification and warming (24ºC and ~1500 µatm pCO_2). Seawater abiotic parameters were daily checked and adjusted to adequate levels whenever needed, as described above. No mortality was observed during the 28 days of trial. By the end of exposure, behavioural tests were conducted (see **section 2.5.**) in ten animals randomly selected out of the three replicates composing each treatment. Afterwards, fish were removed from the test tanks and euthanized with MS222, as previously described. Biometric data were registered (**Table 4.3.1.**), and plasma samples were collected from the 10 fish (as described for Trial I, **section 2.3.1.**) for each treatment (3 tanks per treatment), pooled in two composite samples (n = 2) and kept at -80 ºC until further analyses.

2.4. Venlafaxine determination

Seawater samples were collected from each tank (treatment) in both trials, at days 0, 14 and 28 of the experiment, filtered by PVDF syringe filters 0.22 µm (Merck Millipore) and VFX was quantified by direct injection in UPLC-QqLIT according to the methodology described by Gros et al. (2012). Monitoring VFX concentrations in water along the trials allowed to: a) Trial I - assure a steady VFX concentration throughout the experiment in tanks/treatments simulating VFX exposure via water; b) Trials I and II - assure that no external contamination (apart from the intended dietary exposure) was also taking place in tanks/treatments simulating VFX exposure via feed. VFX concentrations were determined in composite samples of fish plasma (day 28; **Table 4.3.1.**) to establish possible relationships between fish behaviour and VFX

bioaccumulation in the different treatments. For the analysis of VFX in fish plasma, 50 µL of plasma were mixed with 50 µL of methanol and centrifuged (5000 rpm, 10 min, 4 ºC). Then, 60 µL of supernatant were transferred to an insert and 0.6 µL of a 1 ng μL^{-1} VFX-d6 standard solution was added before the analysis by UPLC-QqLIT using the methodology described by Gros et al. (2012). Finally, VFX levels in feed (both, control and VFX-enriched) were determined using an extraction methodology adapted from Jakimska et al. (2013) and further quantified by UPLC-QqLIT by Gros et al. (2012). Data generated was considered satisfactory thanks to the good precision and accuracy of the analytical methodologies previously optimized and validated (Gros et al., 2012; Jakimska et al., 2013), and detailed information on the validation parameters is given in **Annex 7, Table A.7.3**. Venlafaxine net accumulation rates for each treatment (NAR; µg L^{-1} day^{-1}) were determined assuming that fish were exposed to steady conditions (i.e. continuous contaminant exposure, as well as seawater abiotic parameters) and using the following equation (Santana et al., 2017):

$$NAR_t = \frac{([VFX_{t28}] - [VFX_{t0}])}{t}$$

where, $[VFX]_{to}$ is the average VFX concentration in fish plasma before exposure (day 0) and $[VFX]_{t28}$ is the average VFX concentration after 28 days of exposure.

2.5. Behaviour assessment

The first two behaviour tests were conducted according to the Novel Tank assay (to test for anxiety; Test 1) previously described by Egan et al. (2009) and the Shoaling assay (to test social behaviour; Test 2) first described by Moretz et al. (2007), with some modifications (Reyhanian et al., 2011). Briefly, glass test tanks (50 x 26 x 26 cm each) filled with about 20 L of seawater were set up so that Tests 1 and 2 could be performed one after the other, in the same run (the set up for each test can be consulted in **Annex 7, Figure A.7.1**). An isolated zone (8 x 26 x 26 cm), in the right end of each tank, was created using a transparent acrylic plate, to trap and separate a shoal of 5 fish from the testing area. Visual contact beforehand between the test fish and the shoal was avoided by placing a second acrylic plate covered with a black plastic sheet, next to the first plate. Then, the test tanks were horizontally divided, with gridlines marked in the outer part of the tanks, in order to define the bottom (B) and top (T) halves, and vertically divided defining: i) 2 zones for Test 1, i.e. left (L) and right (R) halves; ii) 3 zones for Test 2, i.e. close to the shoal (in_shoal), far from shoal (out_shoal-I) and very far from shoal (out_shoal-II). Before initiating the behaviour assessment, seawater temperature and pH were adjusted according to the experimental conditions set in each treatment (i.e. 19 ºC or 24 ºC, and pCO_2 = ~500 or ~1500 µatm equivalent to 8.0 pH units and 7.6 pH units, respectively), and fish were not fed 12 h prior to the tests. Behaviour tests

were performed by direct observation, using in each test the same team of observers, in order to avoid inter-observer variability. Furthermore, to avoid the potential observer bias, all behaviour tests were performed in a blind way, i.e. no information was provided to the observers regarding the experimental groups that were being tested. In all tests, three test tanks were run at the same time, and behavioural observations were carried out between 8.00 am and 14.00 am, to minimize data variability due to metabolic fluctuations (e.g. cortisol cycle) that normally occur in fish species along the day.

For the Novel Tank assay (Test 1; n = 10), the test fish was gently introduced in the test tank (by netting; 3 s of maximum time outside water), and the counting was initiated as soon as the fish reached the bottom area of the tank. Then, the time spent before crossing, for the first time, the gridline into the top area of the tank (T) was registered, as well as the total time spent in T and the number of vertical grid transitions (from B to T, and from T to B), during 5 min of observation. Fish swimming activity was also evaluated in parallel (counts initiated 30 s after introduction in the test tank), by counting the total number of transitions gridline (horizontal, i.e. from L to R and R to L; and vertical, i.e. from B to T and T to B) during 1 min. After the 5 min time period of Test 1, the Shoaling assay (Test 2; n = 10) was immediately initiated by removing the black acrylic plate (leaving only the transparent one) and, thus, allowing the test fish to visualise the shoal. Then, the time spent before performing, for the first time, a transition towards the shoal (i.e. time to visualise the shoal for the first time) was registered, as well as the total time spent close to the shoal (in_shoal), and the number of gridline transitions towards or away from the shoal (i.e. from out_shoal-I or -II to in_shoal, and vice-versa; out_shoal-I counted as one gridline transitions and outshoal-II counted as two gridline transitions). Test 2 was concluded after a 5 min time period of observation. Fish that exhibited total immobility (i.e. did not show any swimming activity) during the 5 min of tests 1 and 2 were excluded from data analysis (i.e. only three cases: 1 fish from Control treatment, 1 fish from Acid+Warm treatment and 1 fish from Warm+VFX-feed treatment), as they were considered to be in an extreme (unusual) state of physiological stress, which could unlikely be exclusively attributed to the experimental conditions (i.e. VFX exposure, warming and acidification), based on the overall behaviour of the tested group.

Finally, the test fish previously used in Tests 1 and 2 was quickly and gently transferred (by netting; 3 s of maximum time outside water) to another test tank in which the Lateralization Assay (Test 3; n = 10) was carried out (see **Annex 7, Figure A.7.1**), and allowed to acclimate for a period of 5 min (seawater temperature and pH adjusted according to the experimental conditions in each treatment). Test 3 was, then, performed according to the detour test previously described by Bisazza et al. (1998), briefly consisting of a two-way T-maze with a central runway and a movable wall at the end. The test fish was placed in one end of the tank (i.e. the starting point) and compelled to swim forward (by approaching with a scoop, simulating a potential threat). Once

it reached the wall, the fish had to choose which way to turn, i.e. left (L) or right (R), to escape. Ten consecutive runs per test fish were carried out, and the turning side was visually scored. To minimize possible irregularities in the test tank, both ends of the T-maze were alternatively used during the 10 runs. The relative lateralization index (L_R) was calculated for each fish according to Bisazza et al. (1998):

$$L_R = [(\text{turns to the right} - \text{turns to the left}) / (\text{turns to the right} + \text{turn to the left})] \times 100$$

with values close to 100 representing fish that turned right in all 10 runs, −100 representing fish that chose left instead in all 10 runs, and values near zero representing fish that equally preferred left and right. Fish absolute lateralization (i.e. the absolute L_R value for each fish; L_A) was also calculated, with values close to 0 indicating an equal preference for left and right, and values close to 100 indicating a preference for left or right in a total of 10 runs.

2.6. Statistical analysis

To determine significant differences among treatments in VFX plasma concentrations (and NAR), after checking that data complied with assumptions of normality (Kolmogorov–Smirnov's test) and homogeneity of variances (Levene's test), the analysis of variance ANOVA was carried out. Pearson correlation coefficients (*r*) between biometric data and VFX concentrations in fish plasma from each treatment were also calculated. For behavioural data, treatment effects were studied using Generalized Linear Mixed Models (GLMM), with tank replicate as random effect. A Gaussian distribution was used to analyse continuous data (i.e. latency to the top, latency to move towards the shoal and lateralisation), whereas a binomial distribution (or negative binomial when over-dispersion was observed) for proportions was used (percentage of time spent in the top, percentage of time spent within the shoal and percentage of transitions towards the shoal). Moreover, negative binomial distribution was also used for total number of transitions (i.e. fish activity) to account for over-dispersion. Selection for best model was made using Akaike Information Criterion (AIC), and the summary of GLMM results is presented in **Annex 7, Table A.7.4**. Model assumptions, namely independence and absence of residual patterns, were verified by plotting residuals against fitted values and each covariate in the model. The post-hoc Tukey test was also carried out for multiple comparisons (see **Figures 4.3.2.-4.3.4.** and **Table 4.3.2.**). Statistical analysis was performed in R (R Core Team 2017) and data exploration and model validation used the R library from Highland Statistics (Zuur et al 2008). Statistical analyses were performed at a significance level of 0.05.

3. Results

3.1. Biometric parameters and VFX concentrations

Matching the nominal concentration selected for the contaminated feed, VFX concentration in VFX-enriched feed was around 161.7 ± 17.1 µg kg^{-1} dw, whereas VFX was not detected in CTR feed confirming that no external contamination occurred during feed preparation. In seawater samples VFX was only found in detectable levels in VFX-water treatment (day 0: < detection limit, i.e. < 0.15 µg L^{-1}; day 14: 20.9 ± 1.8 µg L^{-1}; day 28: 19.2 ± 1.6 µg L^{-1}), thus confirming that: i) in Trial I, VFX concentration was maintained at around 20 µg L^{-1} in VFX-water treatment throughout the 28 days of exposure; ii) no contamination occurred, apart from the intended contamination of the feed, in VFX-feed, Acid+VFX-feed, Warm+VFX-feed and Acid+Warm+VFX-feed treatments (Trial II).

Biometric parameters (i.e. weight, W, and total length, TL) of fish collected from each treatment in Trials I and II, as well as VFX concentrations in fish plasma are shown in **Table 4.3.1**. In Trial I, W and TL did not significantly vary among treatments ($p > 0.05$), whereas in Trial II, in overall, fish exposed to warmer temperature exhibited significantly higher W and TL ($p < 0.05$), regardless of seawater pH and VFX absence/presence. The maximum values were observed in the Acid+Warm+VFX-feed treatment (weight = 5.0 ± 1.8 g; total length = 8.0 ± 1.2 cm; **Table 4.3.1.**).

No detectable levels of VFX were observed in plasma of control specimens, confirming that there was no other sources of external contamination apart from intended contamination of water (VFX-water treatment; Trial I) or feed (all VFX-feed treatments; Trials I and II). In Trial I, VFX plasma levels were much higher in fish exposed via water compared to fish exposed via feed (~50x higher in VFX-water; $NAR_{VFX\text{-}water}$ = 46 µg L^{-1} day^{-1} against $NAR_{VFX\text{-}feed}$ = 0.5 µg L^{-1} day^{-1}; **Table 4.3.1.**). In Trial II, VFX concentrations in plasma from fish under the control seawater temperature and pH conditions were significantly lower than those in fish exposed to warmer temperature and lower pH simultaneously (i.e. 13.5 ± 1.4 µg L^{-1} against 40.6 ± 11.7 µg L^{-1} in VFX-feed and Acid+Warm+VFX-feed, respectively; $p < 0.05$), but not when fish were exposed to either of the two stressors acting alone (i.e. 24.8 ± 8.5 µg L^{-1} and 34.9 ± 20.6 µg L^{-1} in Acid+VFX-feed and Warm+VFX-feed, respectively; **Table 4.3.1.**). Significantly higher NARs were determined in fish exposed to warming and acidification, acting alone or in combination, compared to those exposed to the control temperature and pH (i.e. VFX-feed; $p < 0.05$; **Table 4.3.1.**). Significant positive correlations were found between W or TL and VFX concentrations, regardless of exposure pathway (W: $r = 0.78$ and $r = 0.70$ for VFX water and feed exposure treatments, respectively; TL: $r = 0.66$ and $r = 0.75$ for VFX water and feed exposure treatments, respectively; $p < 0.01$).

3.2. Behavioural assays

In Trial I, no significant differences between non-contaminated and contaminated fish were observed in the time spent before initiating the exploration of the upper part of the tank (T; **Figure 4.3.2.A**). On the other hand, VFX exposure significantly affected the total time spent within T, regardless of exposure pathway, increasing in fish exposed via water and decreasing in those exposed via feed ($p < 0.05$; **Figure 4.3.2.B**). Activity levels were significantly decreased in VFX contaminated fish (30 ± 12 grid movements in control treatment, against 11 ± 5 and 7 ± 3 in VFX-water and VFX-feed, respectively; $p < 0.05$; **Table 4.3.2.**).

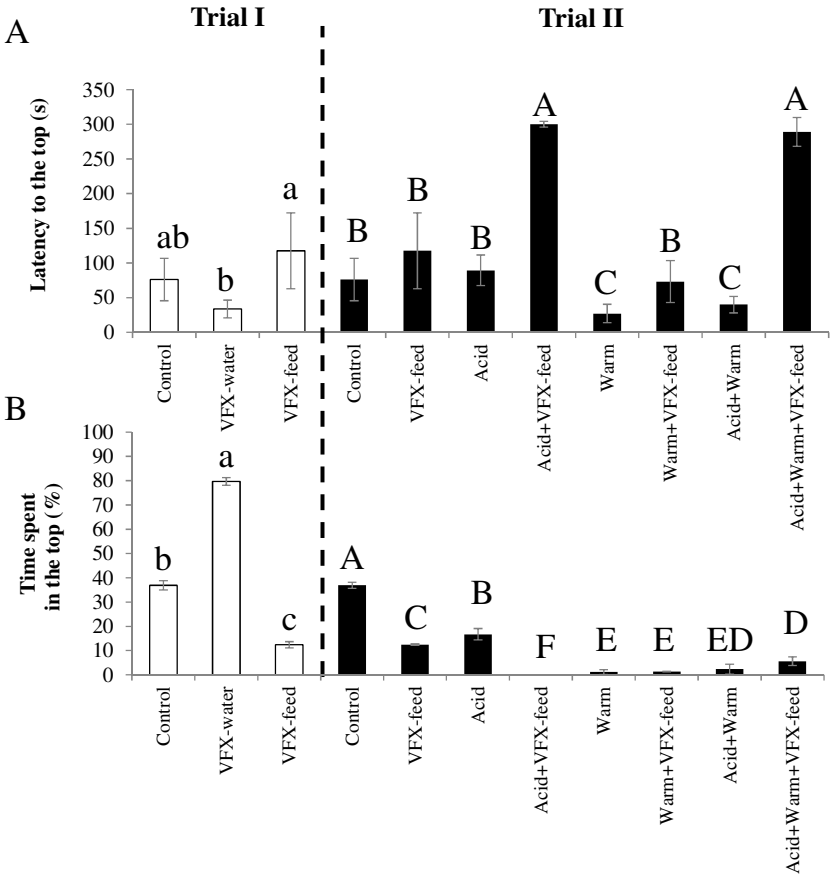


Figure 4.3.2. Latency to reach the top area of the tank, for the first time (A) and percentage of time spent in this area (B), during 5 min of the test ($n = 10$; mean ± standard deviation). Different lower case letters indicate significant differences between treatments in Trial I, whereas upper case letter indicate significant differences between treatments in Trial II ($p < 0.05$). Abbreviations: Acid – simulated acidification (i.e. pCO_2 ~1500 µatm, equivalent to pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC); VFX-water – fish exposed to VFX via water; VFX-feed – fish exposed to VFX via feed.

Table 4.3.2. Number of transitions made to each of 4 halves of the test tank during 1 min of activity test (n = 10; mean ± standard deviation). In each column, different lower case letters indicate significant differences between treatments ($p < 0.05$). Abbreviations: Acid – simulated acidification (i.e. pCO_2 ~1500 µatm, equivalent to pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC); VFX-water – fish exposed to VFX via water; VFX-feed – fish exposed to VFX via feed.

	Total grid movements
Control	30 ± 12^{bc}
VFX-water	11 ± 5^{d}
VFX-feed	7 ± 3^{de}
Acid	80 ± 10^{a}
Acid+VFX-feed	2 ± 2^{e}
Warm	22 ± 4^{c}
Warm+VFX-feed	5 ± 2^{de}
Acid+Warm	45 ± 9^{b}
Acid+Warm+VFX-feed	30 ± 7^{bc}

In the test of social behaviour (shoaling test), despite fish exposed to VFX via feed took less time to perform, for the first time, a transition towards the shoal (**Figure 4.3.3.A**), the percentage of transitions made towards the shoal was not significantly affect by VFX (both exposure routes; **Figure 4.3.3.B**).On the other hand, the total time spent within the shoal was drastically decreased in fish exposed to VFX via water ($p < 0.05$), and even further decreased with VFX exposure via feed ($p < 0.05$; **Figure 4.3.3.C**). Overall, control fish exhibited a preference to turn leftwards (L_R; **Figure 4.3.4.A**). This pattern was maintained in fish exposed to VFX via water, but not in fish from VFX-feed treatment, in which L_R and L_A values closer to zero were observed ($p < 0.05$, for L_R; **Figure 4.3.4.**).

In Trial II, significantly different behavioural patterns were observed in fish exposed to increased temperature and high pCO_2, when acting alone or in combination with VFX exposure (**Figures 4.3.2.-4.3.4.**; **Table 4.3.2.**). Starting with the introduction to a novel environment, acidification combined with VFX exposure significantly increased the time spent before initiating the exploration of the upper areas of the tank (T), regardless of temperature (i.e. treatments Acid+VFX-feed and Acid+Warm+VFX-feed ($p < 0.001$; **Figure 4.3.2.A**). Moreover, fish exposed to VFX, acidification and/or warming tended to spend less time on the upper area of the tank compared to those from Control treatment ($p < 0.05$; **Figure 4.3.2.B**). In terms of overall fish activity, acidification by itself increased the total number of grid transitions in comparison to all the other treatments, whereas in Acid+VFX-feed treatment the number of transitions drastically decreased and no transitions to the top were observed ($p < 0.001$; **Table 4.3.2.**).

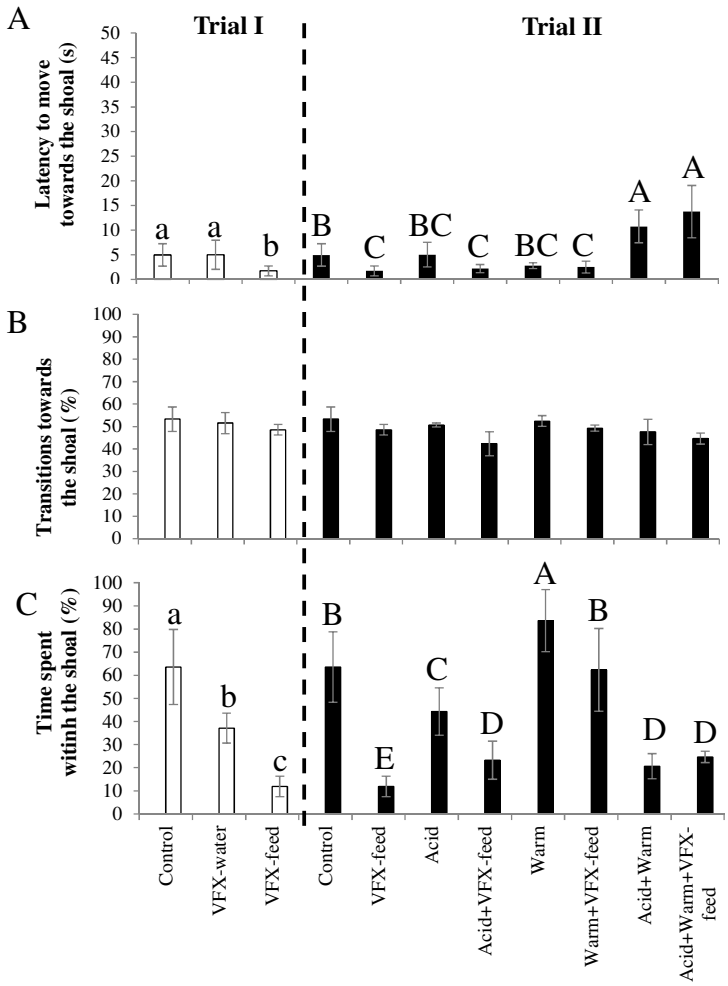


Figure 4.3.3. Time spent until fish visualized the shoal for the first time (A), percentage of transitions towards the shoal (B), and percentage of time spent in this area (C) during the 5 min of shoaling test (n = 10; mean ± standard deviation). Different lower case letters indicate significant differences between treatments in Trial I, whereas upper case letter indicate significant differences between treatments in Trial II (p < 0.05). Abbreviations: Acid – simulated acidification (i.e. pCO_2 ~1500 µatm, equivalent to pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC); VFX-water – fish exposed to VFX via water; VFX-feed – fish exposed to VFX via feed.

During the shoaling test, in both non-contaminated and VFX-enriched fish, warming in combination with acidification increased the time spent before fish performed the first transition towards the the shoal (p < 0.05; **Figure 4.3.3.A**), but not when these two stressors acted independently (regardless of VFX exposure). Despite no significant differences were observed among treatments in the total number of transitions towards the shoal, fish exposed to VFX and/or acidification (i.e. VFX-feed, Acid, Acid+VFX-feed, Acid+Warm and Acid+Warm+VFX-feed) spent significantly less time spent within the shoal formation compared to fish from Control, Warm and Warm+VFX-feed treatments (p < 0.05; **Figure 4.3.3.C**). Furthermore, in general, fish exposed to acidification and/or warming (with and without VFX) tended to stay within the shoal

for a longer period of time than contaminated fish subjected to reference temperature and pCO_2 conditions (i.e. treatment VFX-feed; $p < 0.05$; **Figure 4.3.3.C**).

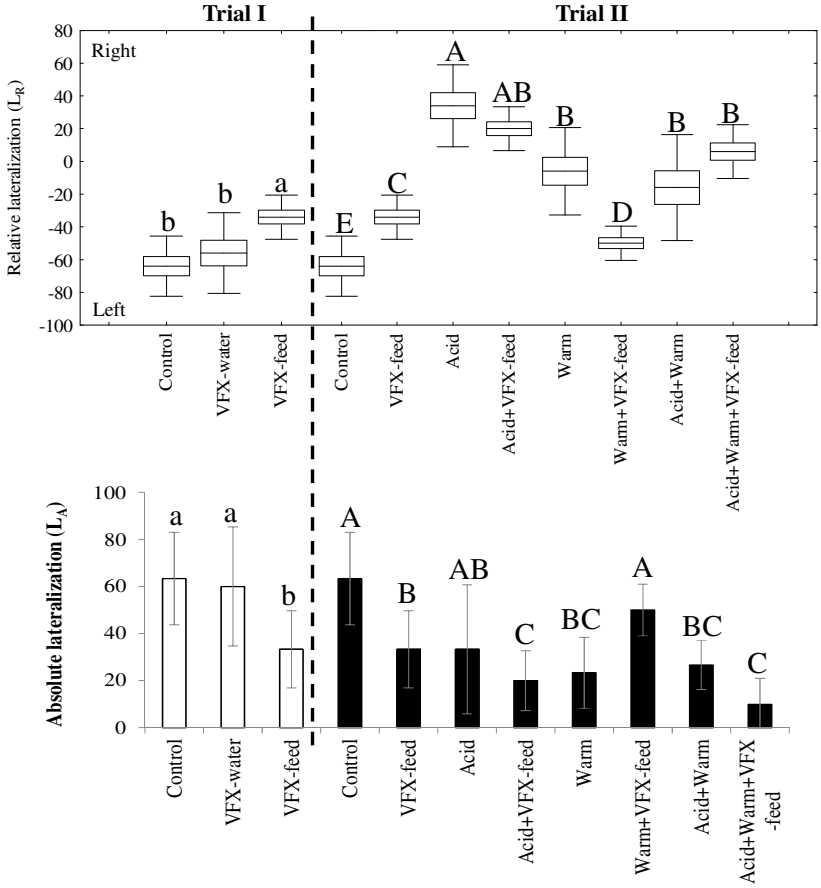


Figure 4.3.4. Relative lateralization (A; L_R; $n = 10$; box plots) and absolute lateralization (B; L_A; $n = 10$; mean ± standard deviation) in *A. regius* after 28 days of exposure to VFX, warming and acidification. Different lower case letters indicate significant differences between treatments in Trial I, whereas upper case letter indicate significant differences between treatments in Trial II ($p < 0.05$). Abbreviations: Acid – simulated acidification (i.e. pCO_2 ~1500 µatm, equivalent to pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC); VFX-water – fish exposed to VFX via water; VFX-feed – fish exposed to VFX via feed.

VFX feed exposure, acidification and/or warming affected fish lateralization, with fish exposed to acidification evidencing a preference to turn rightwards, as opposed to the control, VFX-feed and Warm+VFX-feed treatments in which a turning preference to the left was observed ($p < 0.01$; **Figure 4.3.4.A**). Moreover, an overall loss of preference was observed compared to non-contaminated fish (i.e. lower L_A, with exception of Warm+VFX-feed treatment), particularly in fish exposed to combined acidification and VFX (i.e. Acid+ VFX-feed; $L_A > 20$; $p < 0.01$), as well as when the three stressors were combined (i.e. Acid+Warm+VFX-feed; $L_A \sim 10$; $p < 0.01$; **Figure 4.3.4.B**).

4. Discussion

4.1. Effects of exposure route, temperature and pCO_2 on VFX bioaccumulation

The present results confirmed that VFX can be accumulated, not only from water, but also from diet (and, therefore, biomagnified along the food chain), thus, further emphasizing the ecological hazards this compound can pose to marine ecosystems. Yet, the lower VFX plasma concentration and NAR in fish exposed via feed suggest that diet may indeed play a minor role on the uptake of these ionizable weak base pharmaceuticals, compared to other routes of exposure (such as inhalation), as demonstrated in a recent laboratory study using several aquatic species from different trophic levels exposed to sertraline and fluoxetine (also anti-depressants; Boström et al., 2017). In fish exposed to VFX via feed, increased temperature and pCO_2 levels enhanced VFX bioaccumulation, thus, evidencing the need to consider the potential interactions with abiotic variables when assessing the ecotoxicological implications of pollutants. Such increase in VFX bioaccumulation is most likely related to the metabolic changes induced by altered temperature and pCO_2 (e.g. Rosa et al., 2016; Sampaio et al., 2016, 2018), as well as possible tissue damages, which can then facilitate contaminant penetration into cells (Freitas et al., 2016; Sampaio et al., 2016, 2018; Shi et al., 2016; Velez et al., 2016).

The discrepancy of VFX plasma concentrations and NAR between the two exposure routes may, at a first glance, point out to the favoring of VFX bioaccumulation when exposure occurs via water, a result that is consistent with VFX physical-chemical properties (e.g. log Kow between 2.74 and 3.30; Aryal, 2012). However, it should be noted that the selected VFX concentrations for water and feed are not comparable (and compound bioaccumulation patterns may be dose-dependent) nor the present experimental design allowed to investigate some parameters (e.g. respiration and ingestion rates, compound bioavailability) which are crucial to deeply assess compound toxicokinetics (i.e. such knowledge was outside the scope of this work). Particularly in what concerns VFX exposure via water, the ratio between VFX concentrations in fish plasma and seawater (i.e. the bioconcentration factor, BCF) obtained in the present study (BFC = 64.6 ± 0.4) was much higher than the values previously reported for fish plasma (i.e. BFC = 8; Grabicova et al. (2014) and brain (BCF around 10; Lajeunesse et al., 2011; Grabicova et al., 2014), suggesting that VFX bioaccumulation may be dose dependent. As for the relationship between VFX plasma concentration and fish morphometry, as observed for other chemical contaminants such as MeHg (e.g. Dijstra et al., 2013; Maulvault et al., 2016), results showed that VFX bioaccumulation in fish plasma was directly linked (i.e. correlated) to animal growth, regardless of the exposure pathway. Furthermore, because growth and contaminant metabolization/excretion are also largely influenced by abiotic conditions, changes in seawater temperature and pH can lead to increased contaminant bioaccumulation (e.g. Dijstra et al., 2013; Maulvault et al., 2016;

Sampaio et al., 2016), as observed in Trial II, particularly, when warmer temperatures and high pCO_2 levels were combined.

4.2. Differential effects of VFX exposure route on fish behaviour

Both water and feed exposure triggered significant behavioural alterations, though to different extents and in different directions, and such differences are likely related to the different VFX levels reached in fish plasma in these treatments. Despite the lower concentrations detected in fish plasma, VFX exposure via diet (VFX-feed) significantly affected fish behavior and response to stress, when compared to non-contaminated fish or even to fish exposed to VFX via water (Control and VFX-water). This shows the great ability for this pharmaceutical to easily cross the brain blood barrier (i.e. BCF ~10 in fish brain; Lajeunesse et al., 2011; Grabicova et al., 2014) and promote severe behavioural alterations, even at lower VFX plasma concentrations (Bisesi Jr. et al., 2014), as those elicited by VFX exposure via feed.

Differences between the two exposure routes (i.e. water and feed) were particularly evident in terms of fish exploratory activity and social interactions, which are two ecologically determining factors (Reyhanian et al., 2011; Stewart et al., 2012). Decreased exploratory activity along with increased erratic movements, latency to reach top areas and freezing have been typically associated with increased plasma levels of stress hormones such as cortisol and, consequently, to anxiety in fish (Wibe et al., 2002; Egan et al., 2009). Similarly to the effects induced in both humans and rodents (Katzman, 2004; Sprowles et al., 2017), chronic or acute exposure to different antidepressants has shown to decrease fish anxiety (e.g. citalopram in *Danio rerio*, Sackerman et al., 2010; fluoxetine in *Pimephales promelas*, Margiotta-Casaluci et al., 2014; fluoxetine in *Pachygrapsus crassipes*, Hamilton et al., 2015). Yet, an interesting outcome of the present study was that, in the novel tank test, the anxiolytic effect of VFX was clearly verified in fish exposed to this antidepressant via water (significantly longer permanence in the top area of the tank, despite the lower number of transitions compared to CTR fish), but not in fish exposed to VFX via feed. Such differences between the two contaminated treatments could be related to dose-dependent action of VFX (which is in line with the higher VFX concentrations in plasma of fish exposed via water) and/or to distinct bioavailability of this compound according to the exposure route (Sanchez and Meier, 1997; Brooks et al., 2014; Gray and Hughes, 2015).

Although the apparent state of decreased anxiety in fish exposed to VFX via water (i.e. increased exploratory behaviour) may, at a first glance, come as somewhat positive (e.g. resulting in increased opportunities for feeding, reproduction and territory establishment in the wild), it can also translate into increased risk of predation, which is not beneficial from the ecological point of view. Hence, an increased bottom-dwelling behaviour can be also looked at as an anti-predatory strategy (Maximino et al., 2012). Noteworthy, the increased preference for the bottom of fish

exposed to VFX via feed may also be related to a combination of locomotor (increased sedation) and motivational (anxiolytic-anxiogenic) effects induced by VFX dietary exposure (Maximino et al., 2012; Rosemberg et al., 2012), as it can be corroborated by the lower number of transitions compared to control fish.

Apart from playing a key role as an energy-saving mechanism during swimming, foraging and mating, close shoaling also represents an important anti-predatory strategy, increasing the chances of survival in face of danger, at both the individual and group levels (Pitcher and Parrish, 1993). Here, VFX exposure via water or feed decreased fish tendency to stay within the shoal formation, possibly as a result of lower fish anxiety, which can likely constitute an ecological drawback in the wild (Maximino et al., 2012). A similar trend was also described in study on *D. rerio* exposed to different contaminants with anxiolytic properties (i.e. clonazepam, bromazepam, diazepam, buspirone, propranolol and ethanol; Gebauer et al., 2011). Despite previously evidencing signs of increased anxiety compared to Control and VFX-water treatments, the 5 minutes spent during the first test (novel tank), which worked as an acclimation period before initiating the second test (shoaling), might have contributed to progressively drive fish from VFX-feed treatment into a lower stage of anxiety (i.e. less time spent before performing the first transition towards the shoal compared to Control and VFX-water, as well as similar number of transitions towards the shoal in these three treatments).

The fact that lateralization (due to brain asymmetry) prevails within the animal kingdom suggests that this feature may represent a selective advantage over bilateral control of the cognitive functions (Rogers, 2002; Bisazza and Dadda, 2005). Despite the lack of statistical significance between Control and VFX-water treatments, which could be related to several factors (e.g. individual temperamental characteristics, drug sensitivity, and bias effects specifically associated to the chosen lateralization test, i.e. detour test), the present data suggests that side preference was decreased by VFX exposure via feed. Furthermore, as described by other authors (Bisazza and Dadda, 2005; Bisazza et al., 2000; Bisazza and Brown, 2011), the impairment of side preference promoted by VFX exposure via feed can also be linked to diminished social interactions, thus, matching the diminished time spent within the shoal in VFX-feed treatment, regardless of the number of transitions made on that direction.

4.3. Combined effects of VFX exposure, warming and acidification on fish behaviour

Warming and acidification, alone or combined, significantly enhanced or attenuated the effects of VFX exposure on fish stress response, social skills and lateralization. Elevated pCO_2 levels are known not only to increase animal anxiety and boldness, but also to impair lateralization and olfaction, most likely due to the disruption of the ionic balance in proton-based neurotransmitter receptors, such as $GABA_A$ (e.g. Nilsson et al 2012; Hamilton et al., 2014; Munday et al., 2014;

Sampaio et al. 2016; Lai et al. 2015). For instance, following a light/dark test (scototaxis), Hamilton et al. (2014) reported increased anxiety in juvenile Californian rockfish (*Sebastes diploproa*) exposed to acidification, compared to specimens exposed to normal conditions. Another study using *Atherina presbyter* larvae, also reported decreased shoal cohesion after 7 days of exposure to high pCO_2, as well as individual loss of lateralization (Lopes et al., 2016). Here, in Trial II, significant behavioural changes were observed in fish exposed to acidification, particularly in terms of fish anxiety (less time spent in the top area), activity (increased number of transitions), and lateralization (reversed side preference). Such behavioural effects were further enhanced by VFX exposure via feed, which translated into a substantial decrease of fish swimming activity, exploration and time spent in shoal formation, as well as the loss of side preference that was clearly observed in fish exposed to acidification alone (towards the right). This constitutes an interesting outcome, since VFX acts as an anxiolytic (in humans) and, therefore, a counteraction of the anxiety induced by acidification would be expected instead. Such results further suggest that another mechanism, apart from the altered Cl^- flow through $GABA_A$ receptors one induced by acidification alone, may be involved when VFX and acidification are combined (Nilsson et al., 2012; Hamilton et al., 2014), thus, calling for the need to further explore and understand the neurophysiological mechanisms involved when multiple stressors (such as VFX and acidification) interact.

As for the effects of warming, so far, the majority of studies is primarily focused on metabolic changes and physiological stress induced by thermal stress (e.g. Nilsson et al., 2009; Rosa et al., 2016), whereas little is known regarding its impacts on behavioural cues, such as shoaling and lateralization. Warmer temperatures have been often associated to increased activity and boldness (e.g. Forsatkar et al., 2016). Yet, this was not observed in the present study, as fish exposed to warming exhibited similar swimming activity and spent less time exploring the top area of the tank than Control fish. As for lateralization, a trend similar to the one observed in the present study was also reported by Domenici et al. (2014) in Ward's damselfish (*Pomacentrus wardi)*, with warmer temperatures attenuating the bias observed in control treatments, or even reversing the effects promoted by acidification.

The combination of VFX exposure, warming and acidification seemed to have elicited even more drastic behavioural changes (i.e. increased activity, as well as increased latency for the top area and towards the shoal) compared to each of these stressors acting alone, a result that is aligned with the higher VFX plasma concentration that was also observed in this treatment, thus pointing out to the great ecological impacts involved when the three stressors occur simultaneously. Similarly, in our previous study using juveline flatfish *Solea senegalensis*, distinct behavioural patterns were also observed when three stressors (MeHg exposure, warming and acidification) acted alone or combined, with increased temperatures reversing the effects of acidification in

terms of fish boldness and decision making in non-contaminated fish, whereas such reversion did not occur when MeHg exposure was also added to the equation (Sampaio et al., 2016). To sum up, the present study constitutes a proof of concept that warming, acidification and contaminant exposure can have differential and interactive effects on fish behaviour. Yet, it should be noted that the present findings are limited to the selected levels for VFX exposure and seawater temperature and pCO_2 altered conditions (i.e. only one exposure level was tested for each stressor, given the complexity of the experimental design already as it was) and, therefore, bioaccumulation/behavioural patterns may differ when fish are exposed to a lower or higher severity degree of these stressors.

5. Conclusions

The present study showed that: i) the way, extent and direction in which VFX affects fish behavior is strongly related to exposure route and VFX concentration reached in fish plasma; ii) climate change-related stressors, particularly acidification, significantly affect fish behaviour, which can then translate into deleterious ecological impacts; and iii) such behavioural alterations can be further accentuated or reversed in some instances when these abiotic stressors interact with each other, or when chemical contamination occurs.

The present findings constitute a relevant proof of concept, not only reinforcing the suitability of fish species to assess the toxicokinetics and behavioural implications of SNRI antidepressants, like VFX, but also evidencing the deleterious ecological impacts of human pharmaceutical pollutants on marine vertebrates in tomorrow's ocean. This calls for a better understanding of the ecotoxicological impacts of these compounds on non-target marine vertebrate species, particularly focusing on their different modes of action and bioavailability. Furthermore, the distinct behavioural patterns observed when VFX contamination, high pCO_2 and warming acted alone or in combination also highlight the urgent need to consider multiple environmental stressors (exploring less pronounced to more severe exposure scenarios) in future behavioural ecology studies. Such studies are particularly important since both environmental pollution and climate change effects are expected to worsen in the coming years, and the ecological consequences associated to these stressors, as well as to their interactions with other stressors are still far from being completely understood.

Ethical statement

Fish trials were conducted according to legal regulations (EU Directive 2010/63), and approved by the Ethical Committee of the Faculty of Sciences of the Lisbon University, overseen by the Portuguese National Competent Authority (Direção-Geral de Alimentação e Veterinária, DGAV). All researchers and technicians involved in the maintenance, handling and sampling of

live animals were certified in Laboratory Animal Sciences, by the Federation of European Laboratory Animal Science Associations (FELASA).

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CHAPTER 5.

ENDOCRINE DISRUPTORS: TRICLOSAN AS A CASE STUDY

Parts 1 and 2.

Bioaccumulation and ecotoxicological responses of juvenile white seabream (*Diplodus sargus*) exposed to triclosan, warming and acidification

Manuscript 7.

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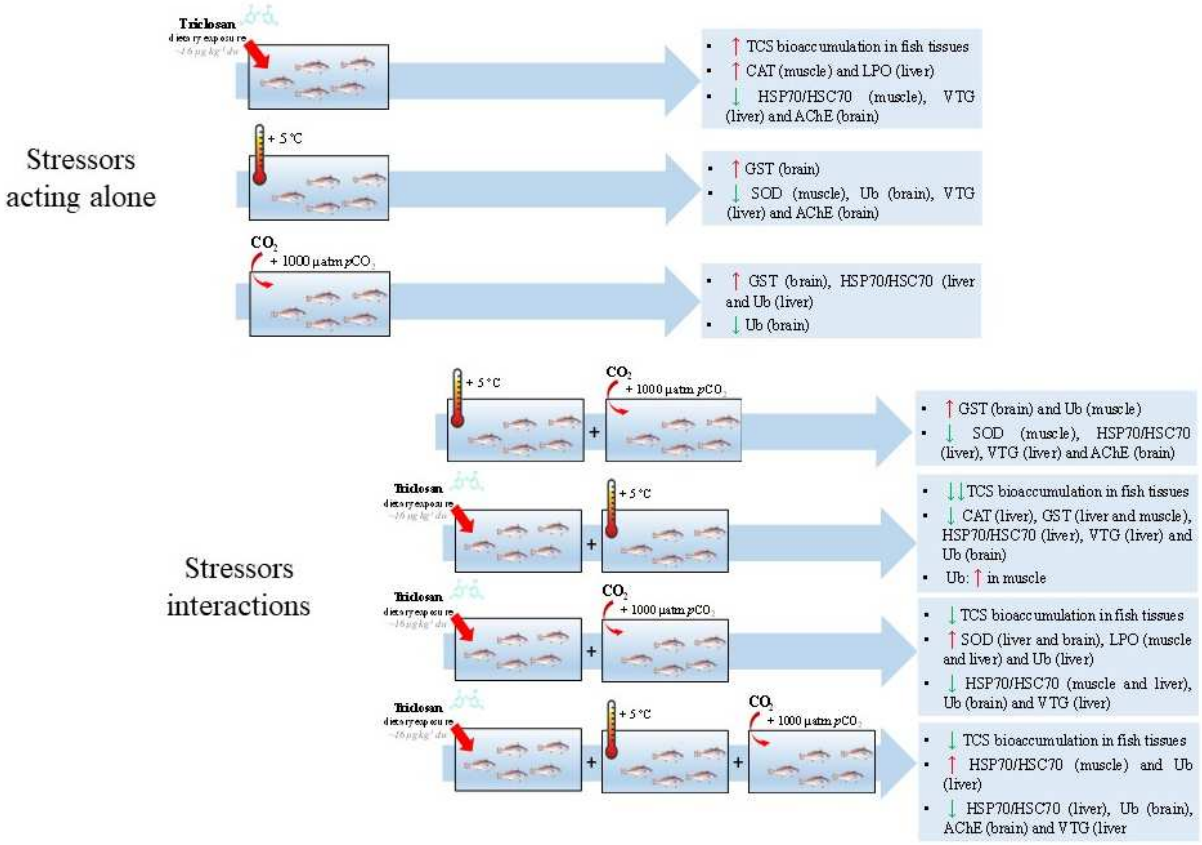
Graphical Abstract



Abstract

Triclosan (TCS) is a synthetic microbial compound widely used in the formulation of various personal care products. Its frequent detection in marine ecosystems, along with its physical and chemical properties, suggest that TCS can be highly persistent, being easily bioaccumulated by biota and, therefore, eliciting various toxicological responses. Yet, TCS's mechanisms of bioaccumulation and toxicity still deserve further research, particularly focusing on the interactive effects with climate change-related stressors (e.g. warming and acidification), as both TCS chemical behaviour and marine species metabolism/physiology can be strongly influenced by the surrounding abiotic conditions. Hence, the aim of this study was to assess TCS bioaccumulation and ecotoxicological effects (i.e. animal fitness indexes, antioxidant activity, protein chaperoning and degradation, neurotoxicity and endocrine disruption) in three tissues (i.e. brain, liver and muscle) of juvenile *Diplodus sargus* exposed to the interactive effects of TCS dietary exposure (15.9 µg kg^{-1} dw), seawater warming (ΔTºC = +5 ºC) and acidification (ΔpCO_2 ~ +1000 µatm, equivalent to ΔpH = -0.4 units). Muscle was the primary organ of TCS bioaccumulation, and climate change stressors, particularly warming, significantly reduced TCS bioaccumulation in all fish tissues. Furthermore, the negative ecotoxicological responses elicited by TCS were significantly altered by the co-exposure to acidification and/or warming, through either the enhancement (e.g. vitellogenin content) or counteraction/inhibition (e.g. heat shock proteins HSP70/HSC70 content) of molecular biomarker responses, with the combination of TCS plus acidification resulting in more severe alterations. Thus, the distinct patterns of TCS tissue bioaccumulation and ecotoxicological responses induced by the different scenarios emphasized the need to further understand the interactive effects between pollutants and abiotic conditions, as such knowledge enables a better estimation and mitigation of the toxicological impacts of climate change in marine ecosystems.

Keywords: Triclosan, bioaccumulation, warming, acidification, multi-biomarkers responses.

1. Introduction

The production and use of pharmaceutical and personal care products (PPCPs) has substantially increased over the last few decades (Arpin-Pont et al., 2016; Rodríguez-Mozaz et al., 2017). As a result, these chemical contaminants are persistently discharged in marine ecosystems (particularly estuaries and coastal areas located near highly urbanized and industrialized areas), through municipal wastewater treatment plants (WWTPs), as well as run offs from agricultural and aquaculture areas (Gros et al., 2012; Gaw et al., 2014). Hence, PPCPs have been frequently detected in marine coastal waters (concentrations ranging from 0.01 ng L^{-1} up to 0.23 mg L^{-1}; Gaw et al., 2014; Arpin-Pont et al., 2016; Rodríguez-Mozaz et al., 2017) and biota (e.g. concentrations ranging from ~1 ng g^{-1} dry weight, dw, for some pharmaceutical active compounds, such as carbamazepine, up to 3600 ng g^{-1} lipid weight, lw, for some preservatives like parabens; Vandermeersch et al., 2015). Yet, PPCPs were only recently regarded as environmental pollutants with potential risks to non-target aquatic species, as well as to humans, and despite some of these compounds have been already included in the list of priority substances in the field of water policy (EC, 2008), their levels in the environment (and seafood) still require regulation.

Triclosan (TCS, 5-chloro-2-(2,4-dichlorophenoxy)phenol) is a synthetic compound widely used in the formulation of various personal care products (e.g. toothpastes, soaps, detergents and cosmetics; EC, 2010, 2014) given its broad-spectrum antibacterial and antimycotic action. In 2016, its production/consumption in the above mentioned products was estimated to be 850 tons in Europe, and 4,760 tons at a global scale (QYR, 2016). Although the high effectiveness of TCS's removal at WWTPs (~90% of the parent compound; TCS usually does not undergo metabolic alteration, since most TCS-containing products are used externally in the human body, and not ingested), small, but not negligible, amounts of TCS are still discharged into the environment through domestic effluents (EC, 2010; Dhillon et al., 2015). Furthermore, agricultural activities are also an (if not the most) important source of TCS environmental contamination, since this compound is also frequently used in the formulation of agro-fertilizing biosolids (Chalew and Halden, 2009). As a result, TCS has been frequently detected in marine ecosystems (e.g. up to 100 ng L^{-1} and 35 μg kg^{-1} dw in seawater and sediments in contaminated coastal areas with strong river and WWTPs discharges, respectively; EC, 2010; from 2 ng g^{-1} dw up to 507 ng g^{-1} dw in marine species; Álvarez-Muñoz et al., 2015; Vandermeersch et al., 2015). Such environmental levels along with TCS chemical properties (i.e. its lipophilic nature) suggest that this pollutant can be highly persistent and easily bioaccumulated by marine species (Dhillon et al., 2015). Apart from its negative effect on microbial resistance, which has been intensively described over the last years (e.g. Bailey et al., 2009; EC, 2010; Jutkina et al., 2018), several ecotoxicological impacts have also been associated with TCS contamination. Some of the previously reported

effects of TCS exposure in aquatic biota include endocrine disruption and neurotoxicity (e.g. Matozzo et al., 2002; Hedrick-Hopper et al., 2015), reduced growth and genotoxicity (Gao et al., 2015), as well as impairments on species' immune system, metabolism and xenobiotic detoxification mechanisms (e.g. Regnault et al., 2016; Mi et al., 2018). Although it is generally assumed that most PPCPs are directly uptaken from water (i.e. through inhalation) and, therefore, most studies using aquatic organisms have focused on water exposure (e.g. Hwang et al., 2014; Rowet et al., 2016; Arnot et al., 2017), TCS chemical attributes (e.g. lipophilicity and logarithm of the octanol-water partition coefficient, log KoW, around 4.8; Dhillon et al., 2015) suggest that diet (i.e. trophic transfer along the food chain) may also represent an important exposure pathway, particularly in predatory fish species (Fair et al., 2009; Zenker et al., 2014; Hedrick-Hopper et al., 2015). Hence, further research is still required to better understand the ecotoxicological impacts of this pollutant to marine organisms, particularly accounting for other compound uptake pathways apart from water exposure.

In parallel with the environmental problematic of pollution, climate change effects (e.g. warming and acidification) in marine ecosystems have also recently raised great concerns among the scientific community and environmental managers/regulators, as changes in seawater abiotic conditions can alter the availability, speciation and toxicity of pollutants (Marques et al., 2010; Maulvault et al., 2016, Sampaio et al., 2018), particularly, ionizable ones, like PPCPs (e.g. Munari et al., 2016; Rowett et al., 2016). Such is the case of TCS, which undergoes protonation and loses its negative charge as the surrounding pH decreases (i.e. ionisation constant, pKa, defined at 8.14; Dhillon et al., 2015), becoming more bioavailable and, thus, toxic (Orvos et al., 2002; Rowett et al., 2016). On the other hand, by affecting marine biotas' physiology and welfare, climate change effects can also potentially compromise the resilience of these species to other stressors, such as chemical contamination (e.g. Rosa et al., 2016; Jesus et al., 2017; Maulvault et al., 2017). Hence, understanding the interactive effects between chemical pollutants and abiotic stressors has become an urgent matter, since climate change effects can already be felt in many geographic locations (e.g. in Ria Formosa coastal lagoon, situated in Olhão, southern Portugal; Barbosa et al., 2010; Rodrigues et al., 2017) and are expected to worsen in the next 50 to 100 years, with southern Europe being particularly prone to these impacts (IPCC, 2001, 2014). According to the most recent projections, seawater temperature and pCO_2 levels are expected to rise as high as +5 ºC and +1000 CO_2 µatm (i.e. equivalent to -0.4 pH units), respectively, even if efforts are made to maintain the emissions of greenhouse gases at the present levels (scenario RCP 8.5, IPCC, 2014; McNeil and Sasse, 2016), likely surpassing species' acclimation ability (i.e. species' physiological and molecular plasticity; e.g. Madeira et al., 2016a; Araújo et al., 2018). Moreover, increased frequency, duration (chronicity) and intensity of extreme climate events, such as heat waves (that last at least 5 days in a row, subsequently, interfering with the remaining water

chemistry parameters), are also expected to occur in a climate change context (IPCC, 2014). These effects are expected to be particularly notorious (or even to be felt in a shorter term) in estuaries and coastal area, which naturally exhibit pronounced shifts of abiotic conditions in month (according to season) or even day time scales (particularly, in shallow water zones with weak hydrodynamic activity, e.g. inner zones of the lagoon and coastal ponds), due to the influence of season and/or tides, among other factors (Barbosa, 2010; Madeira et al., 2016a,b; Rodrigues et al., 2017). Even though marine fish inhabiting these unstable environments (such as the seabreams, e.g. *Diplodus sargus*) may have developed different ecological strategies to cope with great daily or monthly amplitudes of abiotic conditions, recent evidence suggested that these species are extremely vulnerable to climate change effects as they already live close to their physiological thresholds and have limited acclimation plasticity (e.g. Madeira et al., 2012, 2016a). Hence, climate change effects are expected to have devastating impacts in estuaries and coastal areas, like Ria Formosa, causing biodiversity reduction and great economic losses in the fisheries (e.g. due to decreased juvenile recruitment) and aquaculture sectors (Barbosa et al., 2010; Rodrigues et al., 2017), since these ecosystems not only are important spawning and nursery areas (and, thus, shelter many bivalve, crustacean and fish species, e.g. *D. sargus*, during their early and most vulnerable life stages), but also house many aquaculture facilities (in which species are also exposed to changes of abiotic conditions, depending on the type of rearing system used).

Within this context, the aim of the present study was to assess the interactive effects of TCS dietary exposure (15.9 µg kg^{-1} dw), seawater warming ($\Delta T^{o}C$ = +5 ºC) and acidification (ΔpCO_2 ~ +1000 µatm, equivalent to ΔpH = -0.4 units) in compound bioaccumulation and ecotoxicological responses (animal condition, antioxidant activity, chaperoning and protein degradation, neurotoxicity and endocrine disruption) in three tissues (muscle, liver and brain) of juvenile white seabream (*D. sargus*), i.e. an economically and ecologically important fish species that typically inhabits coastal areas subjected to strong anthropogenic and climate-related pressures.

2. Materials and Methods

2.1. Model species

D. sargus is a fish species commonly found in Southern Europe, namely in Ria Formosa coastal lagoon, i.e. a sensitive marine ecosystem (and natural park; ICNF, 2008) exposed to strong anthropogenic and climate-related pressures (as described in the **Introduction section**). It is a highly appreciated seafood species (both wild and farmed) among European consumers, thus, being commercially relevant in the fisheries sector (e.g. about 4000 tons of total catches in 2011; FAO, 2018; up to 29% of total catches in Southern Portugal; IUCN, 2018), as well as in aquaculture (though to a lower extent; i.e. 174 tons produced in aquaculture during 2010; FAO,

2018; IUCN, 2018). By being a predator species that feeds on small benthic organisms that are often in direct contact with contaminated sediments, wild *D. sargus* can likely accumulate high levels of chemical contaminants, particularly through dietary exposure (i.e. ingestion of contaminated preys). As for the effects of abiotic stressors, throughout its life cycle, wild *D. sargus* alternates between estuaries and coastal lagoons during spawning and fish early life stages (eggs, larvae and juveniles), and shallow coastal waters (once the adult stage is reached), therefore, being subjected to wide regimes of seawater abiotic conditions (IUCN, 2018). In aquaculture conditions, although *D. sargus* optimal rearing temperature and pH are around ~19 ºC and ~8.0 pH units, respectively (e.g. Moretti et al., 2005; Saavedra et al., 2006), farmed specimens are also susceptible to strong variations of seawater parameters, since low volumes of water and shallow water depths are usually used in fish rearing. Yet, the severity of these changes will depend on the type of aquaculture system used in fish rearing, as this species can be reared extensively and semi-intensively (in coastal ponds and lagoons), as well as intensively using land-based installations (with open or semi-closed aquaculture rearing systems) or sea cages (Moretti et al., 2005). Based on this background knowledge, *D. sargus* was selected as model species due its ecological and commercial relevance, i.e., it is a commercially relevant predatory fish species (i.e. placed in the higher levels of the food chain) typically exposed to abiotic stressors and pollution and has a very common life cycle among coastal fish species, therefore, representing a very interesting model species that adequately suited the purposes of understanding the ecotoxicological implications of climate change in marine fish.

2.2. Experimental setup

2.2.1. Control and TCS-contaminated feeds

An experimental non-contaminated feed (CTR feed) with an adequate composition to meet the nutritional requirements of juvenile white seabream was manufactured by SPAROS Lda (Olhão, Portugal) (details on feed composition are presented in **Annex 8, Table A.8.1.**). Briefly, all powder components were ground (<200 micron; micropulverizer hammer mill, Hosokawa Micron, SH1, The Netherlands), and mixed with fish oil according to the target feed composition (all ingredients used in fish feeds are presented in **Annex 8, Table A.8.1.**), using a paddle mixer (Mainca RM90, Spain). Then, 25% of deionized water at room temperature was used to further humidify the feed mixture. The feed was subsequently extruded (at 3.0 mm; low-shear extruder, P55, Italplast, Italy) and subsequently dried on vibrating fluid bed dryer (model DR100, TGC Extrusion, France). Then, a 10 kg batch of this non-contaminated feed was also used to perform the enrichment with TCS (i.e. TCS-contaminated feed), by top-coating pellets with a TCS solution ($C_{12}H_7Cl_3O_2$, >98% purity, Sigma-Aldrich; TCS previously solubilized in 5 mL of ethanol and then mixed in fish oil, making up a total volume of 200 mL) using a pressurized spraying container

(standard flat-fan nozzle, size 250 micron, pressure 2.1 bar; the low volume of ethanol used to first solubilize TCS was totally volatilized during this step). Considering the lack of previous studies on pharmaceuticals dietary exposure, a TCS nominal concentration of approximately 15 µg kg^{-1} on a dry weight basis (dw) was selected to assure that TCS bioaccumulation and ecotoxicological responses were induced during the timeline of the trial. As a reference, although such nominal concentration was above the levels frequently found in marine biota inhabiting contaminated coastal areas (i.e. ~7 times higher; Álvarez-Muñoz et al., 2015), this value was still below the lowest observed effect concentration reported in the only available study on TCS dietary exposure in aquatic species, to the best of the authors' knowledge (Hedrick-Hopper et al., 2015). At the beginning and end of the trial, TCS concentration was determined in both experimental feeds in order to assure: i) TCS stability (in TCS-contaminated feed); and ii) no external contamination occurred during feed preparation (in CTR feed).

2.2.2. Specimens collection and acclimation

Juvenile *D. sargus* specimens with similar age (with approximately 200 days of post-hatching period; n = 168) and morphometry (total length: 4.0 ± 0.3 cm; total weight 6.1 ± 0.4 g) were reared at the aquaculture pilot station of the Portuguese Institute for the Sea and Atmosphere (EPPO-IPMA, situated in Ria Formosa coastal lagoon, Olhão, Portugal), using the routine hatchery conditions used in Southern European Countries (e.g. Saavedra et al., 2006). Then, juvenile fish were transplanted (September, 2017, which corresponded to the end of the summer season) to the facilities of Laboratório Marítimo da Guia (MARE-FCUL, Cascais, Portugal), where the exposure trial took place. Here, fish were distributed, in a random and equitable way, in 24 rectangular shaped incubating glass tanks (50 L each, total volume) within recirculation aquaculture systems (RAS; i.e. each group of three tanks corresponded to one treatment, and 3 replicate tanks x 8 treatments = 24 tanks in total; see the description of each treatment in **section 2.3.**) with independent functioning (**Figure 5.1.**). To maintain seawater quality, each tank was equipped with: i) protein skimmers (Reef SkimPro, TMC Iberia, Portugal); ii) ultra-violet (UV) disinfection (Vecton 300, TMC Iberia, Portugal); iii) biological filtration (model FSBF 1500, TMC Iberia, Portugal); and iv) chemical filtration (activated carbon, Fernando Ribeiro Lda, Portugal). Furthermore, fish faeces were removed daily in each incubation tank, and a 25% seawater renewal was performed. Ammonia, nitrite and nitrate levels were daily checked through colorimetric tests (Tropic Marin, USA), and kept below detectable levels (except nitrates, which were kept below 2.0 mg L^{-1}). On a weekly basis, seawater total alkalinity was also measured in every tank according to the methodology of Sarazin et al. (1999), and the carbonate system parameters were calculated through the combination of total alkalinity (AT) and pH (average values obtained for each treatment presented in **Annex 8, Table A.8.2.**).

Before starting the exposure trial, an acclimation period was carried out during 30 days, with fish being fed with CTR feed (2% average body weight, bw) and kept under the following abiotic conditions: i) temperature = 19.0 ± 0.5 °C; ii) pH = 8.00 ± 0.10 units; iii) salinity = 35 ± 1 ‰; iv) dissolved oxygen (DO) > 5 mg L^{-1}; and v) photoperiod = 14 hours light and 10 hours dark (the multi-parameter measuring instrument Multi 3420 SET G, WTW, Germany, was used to daily check seawater temperature, pH, salinity and DO). These seawater abiotic conditions were similar to the ones to which fish were exposed to at EPPO, before being transplanted to the experimental tanks, and corresponded to the optimal conditions for juvenile *D. sargus* rearing (Moretti et al., 2005; Saavedra et al., 2006). Temperature and pH were adjusted whenever needed using: i) temperature - an automatic seawater refrigeration system (± 0.1 °C; Frimar, Fernando Ribeiro Lda, Portugal), as well as submerged digital thermostats (200W, V2Therm, TMC Iberia, Portugal); ii) pH - individual pH probes (GHL, Germany) connected to a Profilux 3.1N control system (± 0.1 pH units;, GHL, Germany), which monitored seawater pH in each tank every 2 s, and adjusted the levels whenever needed, through the injection of either CO_2 (Air Liquide, Portugal; to decrease pH) or CO_2-filtered aeration (Stella 200 air pumps, Aqua One Pro, Aqua Pacific UK Ltd, United Kingdom; to increase pH).

2.2.3. Exposure to TCS, warming and acidification

One week before initiating the trial, seawater temperature and pCO_2 were slowly adjusted (+1 ºC and -0.1 pH unit per day), until reaching the simulated climate change conditions (, i.e. 24 ºC and ~1500 µatm pCO_2, equivalent to pH = 7.6 units, which corresponded to + 5 ºC and +1000 µatm pCO_2 in relation to juvenile *D. sargus* optimal rearing conditions, as well as to the seawater conditions that fish were exposed to before transplantation to the experimental tanks; scenario RCP8.5 of IPCC, 2014; McNeil and Sasse, 2016) in the corresponding treatments (**Figure 5.1.**). Although the seawater warming and acidification conditions simulated in the present study can be defined as an extreme climate change scenario (i.e. worst-case scenario), the reader should note that these conditions are particularly likely to occur (in a shorter time frame) in shallow water coastal environments with low hydrodynamic activity, that naturally exhibit strong tidal/seasonal abiotic variations and are particularly prone to extreme climate events (e.g. heat waves), and in which some of the projected climate change effects are already visible, as is the case of Ria Formosa (Barbosa et al., 2010, Rodrigues et al., 2017). Hence, the altered abiotic conditions simulated in the present study can be considered realistic (according to IPCC projections) and ecologically representative. The remaining seawater abiotic conditions (i.e. DO, salinity and photoperiod) were kept as previously described in **section 2.2.** (i.e. as during the acclimation period). Thus, eight treatments (4 non-contaminated and 4 TCS-contaminated treatments), randomly assigned to each replicate tank, were carried out (n = 7 animals per replicate tank of

treatment, i.e. a total of 21 animals per treatment; **Figure 5.1.**), using a full cross-factorial design: i) Control treatment – 19 ºC, 500 µatm pCO_2 and fish daily fed with CTR feed (2% bw); ii) Acid treatment - 19 ºC, 1500 µatm pCO_2 (equivalent to pH = 7.6 units) and fish daily fed with CTR feed (2% bw); iii) Warm treatment - 24 ºC, 500 µatm pCO_2 and fish daily fed with CTR feed (2% bw); iv) Acid+Warm - 24 ºC, 1500 µatm pCO_2 (equivalent to pH = 7.6 units) and fish daily fed with CTR feed (2% bw); v) TCS treatment – 19 ºC, 500 µatm pCO_2 and fish daily fed with TCS-contaminated feed (2% bw); vi) TCS+Acid treatment - 19 ºC, 1500 µatm pCO_2 (equivalent to pH = 7.6 units) and fish daily fed with TCS-contaminated feed (2% bw); vii) TCS+Warm treatment - 24 ºC, 500 µatm pCO_2 and fish daily fed with TCS-contaminated feed (2% BW); and viii) TCS+Acid+Warm - 24 ºC, 1500 µatm pCO_2 (equivalent to pH = 7.6 units) and fish daily fed with TCS-contaminated feed (2% bw). Seawater physical and chemical conditions were daily monitored and adjusted whenever needed, as described above (**section 2.2.**). No mortality was observed during the 28 day trial. Seawater samples were also collected from each tank at days 0 (beginning) and 28 (end) of the trial, and TCS concentrations were determined to confirm that an external contamination did not occur in non-contaminated treatments, as well as in TCS contaminated treatments (apart from the intended TCS dietary exposure through feed).

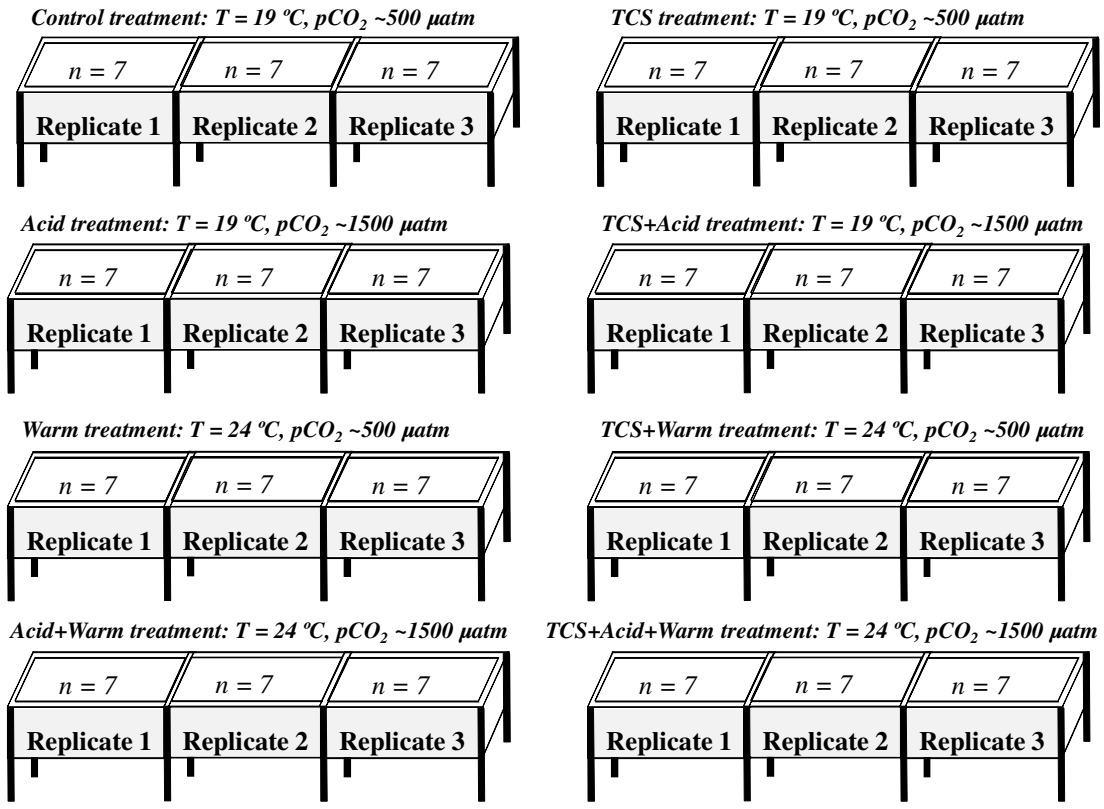


Figure 5.1. Experimental setup. Abbreviations: TCS – triclosan exposure; Acid – simulated acidification (i.e. pCO_2 ~1500 µatm, equivalent to pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC).

2.2.4. Samples collection and preparation

At the end of the trial, 15 fish were randomly collected from each treatment (i.e. 5 fish collected from each of the 3 replicate tanks that composed each treatment), euthanized (i.e. fish immersed for 10 min in a tricaine methanesulfonate, MS222, solution at 2000 mg L^{-1} buffered with sodium bicarbonate, $NaHCO_3$; Sigma-Aldrich, USA for), measured (total length, TL, and weight, W), and dissected (i.e. muscle, brain and liver tissues were collected and weighed).

For TCS determination, due to the amount of sample required by the analytical methodology, two composite samples per tissue and per treatment were performed (i.e. tissues of 5 individuals used in each pool, n = 2 pools). Homogenized pooled samples were freeze-dried (−50 °C, 10^{-1} atm of vacuum pressure for 48 h; Power Dry LL3000, Heto, Czech Republic) and kept at -80 °C until further analysis.

To assess fish ecotoxicological responses, tissues of 5 animals from each treatment were individually used. Each tissue (muscle and liver: ~100 mg; brain: ~40 mg) was homogenized with 1.0 mL of phosphate buffered saline (PBS; 3mM of potassium chloride, KCl, 10 mM of potassium phosphate monobasic, KH_2PO_4 and 140 mM of sodium chloride, NaCl, pH adjusted to 7.40 ± 0.02 units; all reagents from Sigma-Aldrich, Germany), in ice-cold conditions, using a T25 digital Ultra-Turrax device (Ika, Germany). Crude homogenates were then centrifuged in 1.5 mL Eppendorf tubes (15 minutes, 10.000 *g*, 4 ºC), and supernatants were transferred to new Eppendorf tubes, being immediately frozen and kept at -80 ºC until further analyses.

2.3. TCS determination in seawater, feeds and fish tissues

For TCS determination in liquid samples (i.e. seawater), the protocol followed was based on the methodology described by Castro et al. (2018), with some adjustments. Briefly, 4.0 mL of sample was placed in 10 mL conic glass tube, and 100 µL of Chrysene D-12 at 0.5 mg L^{-1} were added (solution pH adjusted to ≥ 10 pH units, using droplets of potassium carbonate, K_2CO_3, 23%, analytical grade, Panreac Química SA). Then, a mixture of 85 µL of carbon tetrachloride (CCl_4; 99.9% purity, Panreac Química), 800 µL of acetonitrile and 125 µL of acetic anhydride was rapidly transferred to the 10 mL tube. Each tube was vortexed for 1 minute and centrifuged at 1500 *g* for 3 minutes. Then, 50 µL of end-phase was transferred to an injection vial containing 10 µL of methyltriclosan D-3 at 2 mg L^{-1}, and 1 µL of this mixture was injected into the gas chromatography–mass spectrometry (GC-MS) system.

In solid samples (i.e. feeds and fish tissues), a combined QuEChERS (quick easy cheap effective rugged safe) and DLLME (dispersive liquid-liquid micro extraction) procedure was first carried out following the methodology of Castro et al. (2018) with some modifications. Briefly, 500 mg of feed or freeze-dried tissue sample was weighted to 40 mL dark glass tubes, and 50 µL

of internal standard (IS1) was added (Chrysene D_{-12}, 3 mg L^{-1}; 98 atom %, Sigma-Aldrich). After 15 min, samples were hydrated with deionized water (5 mL), and 4 mL of acetonitrile (MeCN, ≥ 99.9% purity, Honeywell Riedel-de Haën ACS), 0.5 g of NaCl (≥ 99.9% purity Sigma-Aldrich) and 2 g of anhydrous magnesium sulphate ($MgSO_4$, ≥ 99.5%, Sigma-Aldrich) were added. Sample tubes were vortexed for 30 seconds, placed in a round shaker for 30 minutes and, then, centrifuged again (5 minutes at 4000 *g*). The extracts obtained were used as a dispersive solvent for DLLME. Hence, to 1 mL of MeCN extract obtained, 50 µL of internal standard (IS2; methyl-triclosan D3, 2 mg L^{-1}, Sigma-Aldrich), 60 µL of CCl_4, and 125 µL of acetic anhydride ($C_4H_6O_3$, ≥99% purity, Sigma-Aldrich) were added. This mixture was quickly transferred to a 10 mL screw cap glass tubes with a conical bottom containing 3 mL of deionized (pH ≥ 10 units, adjusted with K_2CO_3 23% droplets). Tubes were manually shaken for a few seconds and centrifuged (3 minutes at 4000 *g*). The settled volume (60 ± 5 µL) was transferred to an injection vial with 10 µL of methyltriclosan D-3 at 2 mg L^{-1}, and 1 µL of sample was injected in the GC–MS system.

GC-MS analyses were carried using an Agilent gas chromatograph 6890 (Little Falls, DE, USA) equipped with an electronically controlled split/splitless injection port and interfaced to a MSD-5975B mass-selective detector. GC separation was conducted on a DB-5MS column (30 m × 0.25 mm i.d. 0.25 µm film thickness; J&W Scientific, Folsom, CA, USA), using helium as the carrier gas (1 mL $minute^{-1}$ of constant gas flow). Injections were made in splitless mode (purge-off time: 60 s) at 250 °C, and the following oven temperature programme (with 28 min of total run time) was carried out: 1 minute hold at 90 °C, temperature ramped at 20 °C $minute^{-1}$ until 150 °C, 5 °C $minute^{-1}$ between 150 ºC and 225 °C, and then 20 °C $minute^{-1}$ between 225 ºC and 300 °C, 5.25 minute hold. The following mass spectrometric parameters were set: 70 eV energy electron ionization, 230 °C of ion source temperature, and 150 °C of mass spectrometry (MS) quadrupole temperature. Selective ion monitoring (SIM) mode was employed and each analyte was quantified using the peak area of one target ion and two qualifier ions. Data collection/processing and GC-MS control was performed on the software Agilent Chemstation. SIM parameters, retention times of analytes, and validation parameters of the methodology are shown in **Annex 8, Table A.8.3**.

2.4. *D. sargus* ecotoxicological responses

2.4.1. Fitness indexes (K, HSI and BB_{ratio})

Animal condition was determined by calculating the Fulton's K index (Ricker, 1975), using fish weight (W) and total length (TL):

$$K = 100\ x\ \frac{W\ (g)}{TL^3(cm)}$$

Organ condition (liver and brain) was also assessed by calculating the hepatosomatic index (i.e. HSI; Diniz et al., 2009) and brain to body mass ratio (i.e. BB_{ratio}; Maulvault et al., 2017) using the following equations:

$$HSI = \frac{Liver\ weight\ (g)}{W\ (g)}\ x\ 100$$

and,

$$BB_{ratio} = \frac{brain\ weight\ (g)}{W\ (g)}\ x\ 100$$

2.4.2. Molecular biomarkers

Eight molecular biomarkers (of exposure and/or effect) were selected to assess distinct biological effects (endpoints) induced by TCS dietary exposure, warming and acidification, at the tissue level (**Table 5.1.**). These molecular biomarkers have been widely employed in ecotoxicological studies, being considered as reliable and suitable to assess the effects of xenobiotics exposure, including TCS (e.g. Matozzo et al., 2012), as well as of climate change-related effects (e.g. Rosa et al., 2016; Jesus et al., 2017; Maulvault et al., 2017, 2018a). **Table 5.1.** presents a summary of the selected biomarkers (and the corresponding ecotoxicological response tested), as well as the different methodologies used (further details regarding these methodologies have been previously presented in Maulvault et al., 2017 and Maulvault et al., 2018a). All biomarker values were normalized using the total protein content of each sample (i.e. results presented in mg of protein), determined through the Bradford assay (Bradford, 1976).

All protocols used for both enzymatic and protein quantification assays were adapted to 96-well microplates (microplates from Nunc-Roskilde, Denmark, used in all assays), and carried out in triplicate, and read in a Multiskan Go 1510 microplate reader (ThermoFisher Scientific, USA). All reagents used were of pro analysis grade or higher.

Table 5.1. Summary of selected molecular biomarkers and the corresponding methodologies used.

Biomarkers	Category	Tested ecotoxicological response	Type of methodology used	Methodology references
Catalase (CAT) activity	Biomarker of effect	Oxidative stress	Enzymatic assay	Johansson and Borg (1988); Maulvault et al. (2017)
Superoxide dismutase (SOD) activity	Biomarker of effect	Oxidative stress	Enzymatic assay	Sun et al. (1988); Maulvault et al. (2017)
Glutathione S-transferase (GST) activity	Biomarker of effect	Oxidative stress and xenobiotic detoxification phase II	Enzymatic assay	Habig et al. (1974); Maulvault et al. (2017)
Lipid peroxidation (LPO)	Biomarker of effect	Oxidative stress and cellular damage	TBARS method	Uchiyama and Mihara (1978); Madeira et al. (2016a)
HSP70/HSC70 proteins content	Biomarker of effect	Chaperoning, heat shock response	Indirect ELISA	Njemini et al. (2005); Maulvault et al. (2017)
Total Ubiquitin (Ub) content	Biomarker of effect	Protein degradation and DNA repair	Direct ELISA	Madeira et al. (2014)
Acetylcholinesterase (AChE) activity	Biomarker of effect and exposure	Neurotoxicity	Enzymatic assay	Ellman et al. (1961); Maulvault et al. (2017)
Vitellogenin (VTG) content	Biomarker of exposure	Endocrine disruption and reproduction	Direct ELISA	Denslow et al. (1999); Diniz et al. (2009)

2.5. Statistical analyses

For the different studies variables, differences between treatments were investigated using Generalized Linear Mixed Models (GLMM). Selection for best model was made using Akaike Information Criterion (AIC), i.e.: a Gamma distribution was used to analyse TCS tissue concentration data, whereas a Gaussian distribution was used for fish morphometric (W, TL) and ecotoxicological data (K, HSI and BB_{ratio}, as well as tissue biomarker responses), with replicate tank as random effect. Model assumptions, namely independence and absence of residual patterns, were verified by plotting residuals against fitted values and each covariate in the model. The summary of GLMM results is presented in **Annex 8, Tables A.8.4.-A.8.6**. The post-hoc Tukey test was also carried out for multiple comparisons (results are presented in **Figures 5.2.-5.6.**). Finally, Pearson correlation coefficients between variables were also calculated. Statistical analyses were performed at a significance level of 0.05, and using the R Core Team 2017 software. Data exploration and model validation used the R library from Highland Statistics (Zuur et al., 2009).

3. Results

3.1. TCS concentration in feeds and seawater samples

An average TCS concentration of 15.9 ± 2.9 µg kg^{-1} dw was obtained in TCS-contaminated feed throughout the trial, whereas TCS was not detected in CTR feed (i.e. CTR feed concentration < 0.009 µg kg^{-1}). In the beginning of the trial (day 0), seawater samples from all treatments revealed TCS below LOD (i.e. concentration < 0.050 µg L^{-1}). Yet, at day 28, quantifiable and similar TCS concentrations were found in contaminated treatments (average concentration = 0.44 ± 0.10 µg L^{-1}; **Figure 5.2.A**), while TCS levels remained below LOD in the Control treatment.

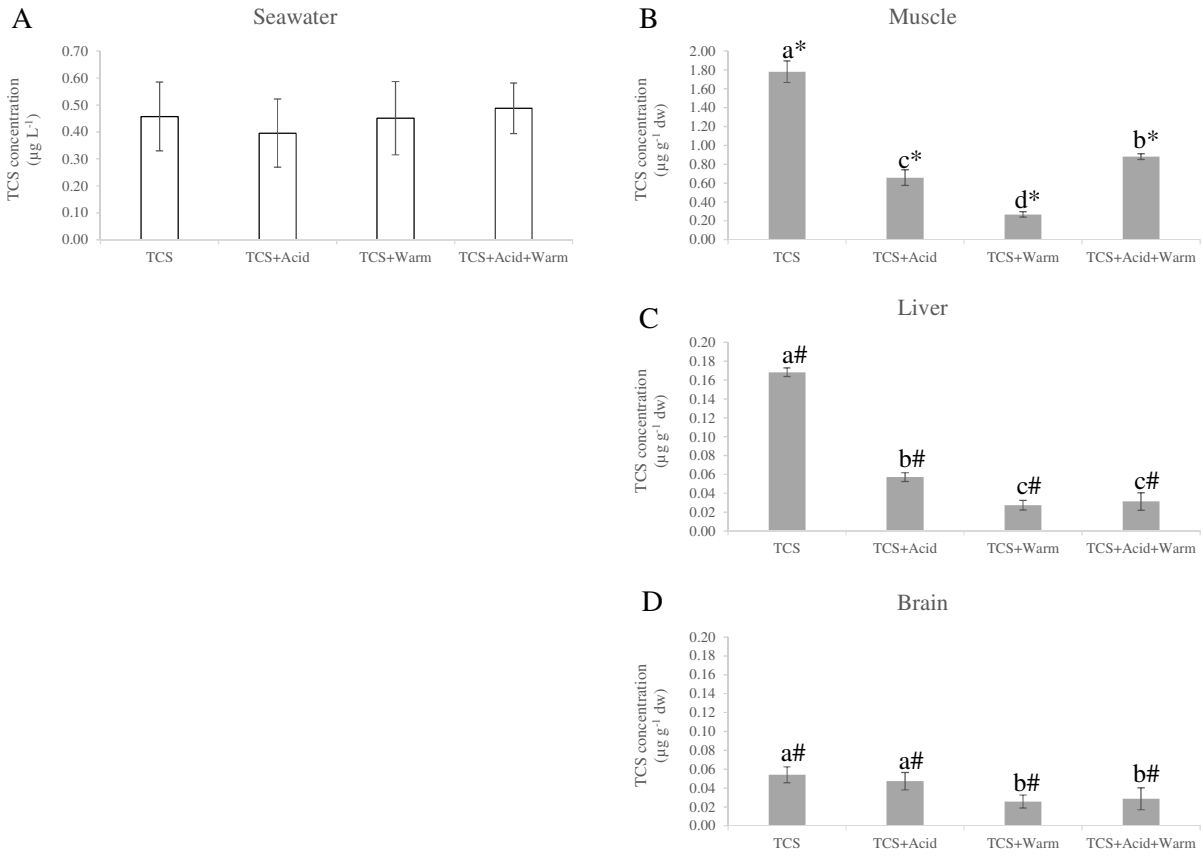


Figure 5.2. Triclosan concentration in seawater samples (µg L^{-1}; A) and in fish tissues (µg kg^{-1} dw; B-D) from contaminated treatments after 28 days of TCS dietary exposure. Different letters (a-d) indicate significant differences between treatments (for the same tissue; $p < 0.05$), whereas different symbols (*, # or /) indicate significant differences between tissues (for the same treatment; $p < 0.05$). Abbreviations: TCS – triclosan exposure; Acid – simulated acidification (i.e. pCO_2 ~1500 µatm, equivalent to pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC).

3.2. Fish morphometry and TCS tissue bioaccumulation

After 28 days of trial, fish exposed to the reference temperature and pCO_2 levels (i.e. Control and TCS treatments) exhibited significantly lower W and TL than fish exposed to warming alone (i.e. Warm treatment; $p < 0.010$) or in combination with TCS exposure and acidification (i.e. TCS+Acid+Warm treatment; $p < 0.050$; **Figure 5.3.A**).

By the end of the exposure trial, none of the tissues of non-contaminated fish (i.e. Control treatment) revealed detectable levels of TCS (i.e. concentration in fish tissues < 0.009 µg kg^{-1}). As for contaminated treatments, TCS concentrations significantly varied among tissues and treatments, with muscle being the primary organ for TCS bioaccumulation regardless of seawater temperature and pCO_2 conditions (e.g. in TCS treatment: 1.781 ± 0.114 µg kg^{-1} dw in muscle, 0.168 ± 0.005 µg kg^{-1} dw in liver and 0.050 ± 0.009 µg kg^{-1} dw in brain; $p < 0.001$; **Figures 5.2.B-D**). Furthermore, significantly lower TCS concentrations were found under increased seawater temperature and/or pCO_2 levels, regardless of tissue, with warming overall yielding lower TCS

levels in all organs (i.e. TCS+Warm treatment: 0.268 ± 0.027 µg kg^{-1} dw in muscle, 0.027 ± 0.005 µg kg^{-1} dw in liver and 0.026± 0.007 µg kg^{-1} dw in brain) compared to the reference seawater temperature conditions or to acidification alone (**Figures 5.2.B-D**). Significant negative correlations were obtained between morphometric data and TCS concentration in liver (r = -0.62 and r = -0.55 and p < 0.010 and p < 0.050 in W and TL *versus* TCS liver concentration, respectively; Table 2) and brain (r = -0.60 and r = -0.53 and p < 0.01 and p < 0.050 in W and TL *versus* TCS brain concentration, respectively; **Table 5.2.**) of contaminated fish, but not in muscle (i.e. p > 0.050; **Table 5.2.**).

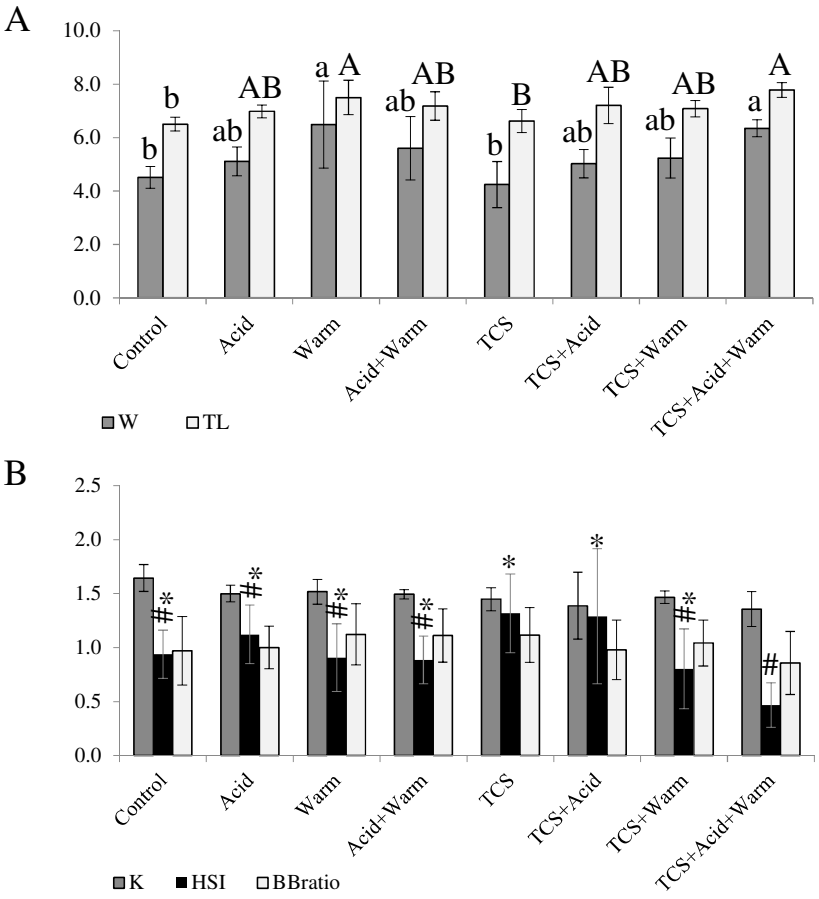


Figure 5.3. *D. sargus* total length (TL; cm) and weight (W; g), Fulton's condition index (K), hepatosomatic index (HSI) and brain-to-body mass ratio (BB_{ratio}) after 28 days of trial (mean ± SD; n = 15). Different letters (lower case, a-b, in W bars and upper case, A-B, in TL bars) and symbols (* or # in HSI bars) indicate significant differences (p < 0.05) between treatments. Abbreviations: TCS – triclosan exposure; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC).

Table 5.2. Pearson correlation coefficients (*r*) between TCS tissues concentration, animal morphometry, fitness indexes and molecular biomarker responses. In each column, asterisks indicate significant correlations between variables (*: $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Abbreviations: TW – total weight; TL – total length; K - Fulton's condition index; HSI - hepatosomatic index; BB_{ratio} - brain-to-body mass ratio; CAT – catalase activity; SOD activity inhibition – superoxide dismutase; GST activity – glutathione S-transferase; LPO - lipid peroxidation; HSP70/HSC70 - heat shock proteins concentration; Ub - total ubiquitin concentration; AChE – acetylcholinesterase activity; VTG – vitellogenin concentration; TCS – triclosan exposure; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC).

Treatments used in the analysis	Variables	*r*		
All	**W x TL**	0.85***		
	K x HSI	0.19		
	K x BB_{ratio}	0.05		
		Muscle	**Liver**	**Brain**
TCS-contaminated treatments	**[TCS] x W**	-0.41	-0.62**	-0.60**
	[TCS] x TL	-0.38	-0.55*	-0.53*
	[TCS] x K	0.04	0.08	0.12
	[TCS] x HSI	-	0.46*	-
	[TCS] x BB_{ratio}	-	-	0.37
	[TCS] x CAT	0.77***	-0.02	-0.17
	[TCS] x SOD	-0.20	-0.001	-0.20
	[TCS] x GST	0.26	0.09	0.19
	[TCS] x LPO	0.11	0.17	-0.40
	[TCS] x HSP70/HSC70	-0.17	0.58**	-0.02
	[TCS] x Ub	-0.40	0.47*	0.51*
	[TCS] x AChE	-	-	-0.07
	[TCS] x VTG	-	-0.21	-
All	**K x CAT**	0.01	-0.16	-0.04
	K x SOD	-0.10	0.09	0.19
	K x GST	0.13	-0.04	-0.07
	K x LPO	-0.02	-0.19	-0.02
	K x HSP70/HSC70	-0.09	0.27	0.40**
	K x Ub	-0.14	-0.33*	0.22
	K x AChE	-	-	0.12
	K x VTG	-	0.37*	-
All	**HSI x CAT**	-	-0.17	-
	HSI x SOD	-	-0.31	-
	HSI x GST	-	0.24	-
	HSI x LPO	-	0.28	-
	HSI x HSP70/HSC70	-	0.19	-
	HSI x Ub	-	-0.19	-
	HSI x AChE	-	-	-
	HSI x VTG	-	0.11	-
All	**BB_{ratio} x CAT**	-	-	-0.07
	BB_{ratio} x SOD	-	-	0.05
	BB_{ratio} x GST	-	-	0.28
	BB_{ratio} x LPO	-	-	-0.17
	BB_{ratio} x HSP70/HSC70	-	-	-0.19
	BB_{ratio} x Ub	-	-	-0.04
	BB_{ratio} x AChE	-	-	-0.12
	BB_{ratio} x VTG	-	-	-

3.3. Ecotoxicological responses

Fish fitness indexes (K, HSI and BB_{ratio}) after 28 days of exposure to the three stressors are presented in **Figure 5.3.B**. While K and BB_{ratio} were not significantly affected by TCS, acidification and/or warming (i.e. $p > 0.050$ in Control treatment against all treatments), significantly higher HSI was found in fish exposed to TCS alone or in combination with acidification compared to fish exposed to the three stressors simultaneously ($p < 0.010$ and $p < 0.05$, respectively; **Figure 5.3.B**). Moreover, HSI was significantly and positively correlated with TCS liver concentrations in contaminated fish ($r = 0.46$ and $p < 0.050$; **Table 5.2.**).

Figure 5.4. presents the antioxidant enzymes activity (CAT, SOD and GST) and lipid peroxidation (LPO) in fish tissues. In fish muscle, a significant increase in CAT activity was observed when TCS exposure acted alone (corresponding to a 54.5% increase in relation to Control treatment; $p < 0.050$), but not when combined with the warming and/or acidification (**Figure 5.4.A** and **Annex 8, Table A.8.7.**). Furthermore, in the muscle of contaminated fish, a significant and positive correlation was found between CAT activity and TCS concentration ($r = 0.77$ and $p < 0.001$; **Table 5.2.**). As for the liver and brain, although CAT activity was not significantly affected by the three stressors acting alone ($p > 0.05$), a significant decrease was observed in the liver of fish co-exposed to TCS and warming (i.e. TCS+Warm treatment), not only in relation to the Control treatment (equivalent to a 38.7% decrease; $p < 0.001$), but also to Warm ($p < 0.001$), TCS+Acid ($p < 0.001$) and TCS+Acid+Warm treatments ($p < 0.001$; **Figure 5.4.B** and **Annex 8, Table A.8.7.**). SOD and GST activities were significantly affected by abiotic stressors, acting alone or combined with each other (i.e. SOD muscle activity decreased around 9% in relation to Control treatment; ; $p < 0.050$ and $p < 0.010$, in Control against Warm and Acid+Warm treatments, respectively; GST brain activity increased between 76% and over 100% in relation to Control treatment; $p < 0.050$, $p < 0.001$ and $p < 0.001$ in Control against Acid, Warm and Acid+Warm treatments; **Figures 5.4.D-I** and **Annex 8, Table A.8.7.**). On the other hand, TCS exposure did not affect SOD activity, unless in the liver and brain of fish exposed to the combination of TCS and acidification (corresponding to activity increases of 18.6% and 5.5% in relation to Control treatment, in liver and brain, respectively; $p < 0.001$ and $p < 0.050$, respectively; **Figures 5.4.E-F** and **Annex 8, Table A.8.7.**). Similarly, TCS exposure did not affect GST activity, except in the muscle and liver of fish co-exposed to TCS and warming (i.e. corresponding to a decrease of 46.8% and 69.6% in relation to Control treatment in muscle and liver, respectively; $p < 0.001$ in both cases; **Figures 5.4.G-H**, and **Annex 8, Table A.8.7.**). It should be further noted that significantly higher GST activity was also found in TCS+Acid treatment compared to TCS+Warm treatment (in muscle and liver; $p < 0.001$ in both cases), as well as to TCS and TCS+Acid+Warm treatments (in liver; $p < 0.001$ and $p = 0.019$, respectively; **Figures 5.4.G-.H**). Overall, TCS acted alone or in combination with acidification induced

significantly higher LPO in fish muscle and liver (i.e. average increase of over 100% in relation to Control treatment, in both tissues), but not in the brain ($p > 0.050$; **Figures 5.4.J-L** and **Annex 8, Table A.8.7.**).

HSP70/HSC70 and Ub contents in the different fish tissues are shown in **Figure 5.5.** Contrasting the trends mentioned above, in the absence of TCS (i.e. in non-contaminated fish), abiotic stressors only affected HSP70/HSC70 content in fish liver, resulting in a significant increase in Acid treatment (i.e. equivalent to 63.2% increase in relation to Control treatment; $p < 0.001$), whereas Acid+Warm treatment significantly inhibited the synthesis of these proteins (i.e. equivalent to 74.0% decrease in relation to Control treatment; $p < 0.001$; (**Figures 5.5.A-C** and **Annex 8, Table A.8.7.**). Conversely, TCS alone or combined with acidification significantly lowered HSP70/HSC70 contents in fish muscle (corresponding to a 44.6% and 56.6% decrease in TCS and TCS+Acid treatments, respectively, relation to Control treatment; $p < 0.001$ in both cases), but such effect was reversed when the three stressors acted simultaneously (i.e. in TCS+Acid+Warm treatment; corresponding to an increase of 66.5% in relation to Control treatment; $p < 0.001$; **Figure 5.5.A** and **Annex 8, Table A.8.7.**). In fish liver, significant effects were only found when TCS was combined with abiotic stressors (i.e. decreases in HSP70/HSC70 contents between 36.6% in TCS+Warm treatments and 76.6% in TCS+Acid treatment, relation to the Control treatment; $p < 0.010$ and $p < 0.001$, respectively; **Figure 5.5.B** and **Annex 8, Table A.8.7.**). No significant differences were observed in HSP70/HSC70 brain contents ($p > 0.050$; **Figure 5.5.C** and **Annex 8, Table A.8.7.**). A significant and positive correlation was found between HSP70/HSC70 and TCS contents in fish liver ($r = 0.58$ and $p < 0.001$), as well as between HSP70/HSC70 and K in the brain ($r = 0.40$ and $p < 0.001$; **Table 5.2.**). As for Ub content, warming combined with acidification or with TCS significantly increased Ub protein synthesis in fish muscle (i.e. 85.4% and 66.0% increase in relation to Control treatment in Acid+Warm and TCS+Warm treatments, respectively; $p < 0.001$ in both cases; **Figure 5.5.D** and **Annex 8, Table A.8.7.**). On the other hand, Ub contents in fish liver were significantly increased in acidified conditions (i.e. average increase of over 100% in relation to the Control treatment for TCS+Acid and TCS+Acid+Warm treatments; **Figure 5.5.E** and **Annex 8, Table A.8.7.**). In the brain, with the exception of Acid+Warm and TCS treatments, all other treatments significantly reduced Ub protein synthesis in relation to the Control treatment, particularly with acidification acting alone (which yielded a 69.5% decrease in relation to Control treatment; $p < 0.001$; **Figure 5.5.F** and **Annex 8, Table A.8.7.**). Ub contents in both liver and brain of contaminated fish were significantly correlated with TCS contents ($r = 0.47$ and $r = 0.51$, respectively, and $p < 0.050$ in both cases), as well as to K (only in liver; $r = -0.33$ and $p < 0.050$; **Table 5.2.**).

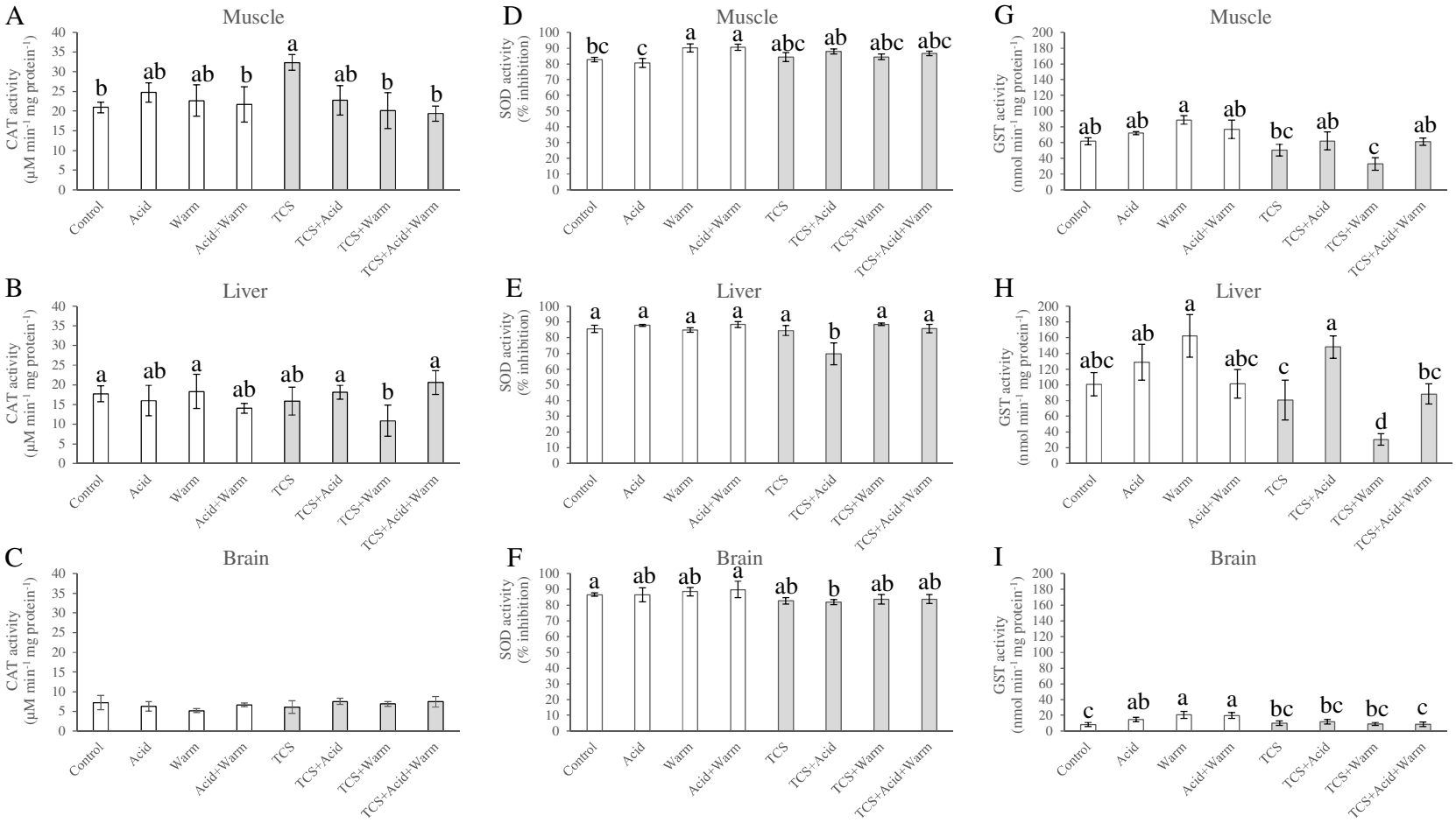


Figure 5.4. Antioxidant activity (CAT, µM min $^{-1}$ mg^{-1} protein; SOD, % inhibition; GST, nmol min $^{-1}$ mg^{-1} protein) and lipid peroxidation (expressed as MDA concentration; µM mg^{-1} protein) in muscle (A, D, G, J), liver (B, E, H, K) and brain (C, F, I, L) of *D. sargus* after 28 days of exposure (mean ± SD; n = 5). Different letters (a-c) indicate significant differences between treatments (for the same tissue; $p < 0.05$).

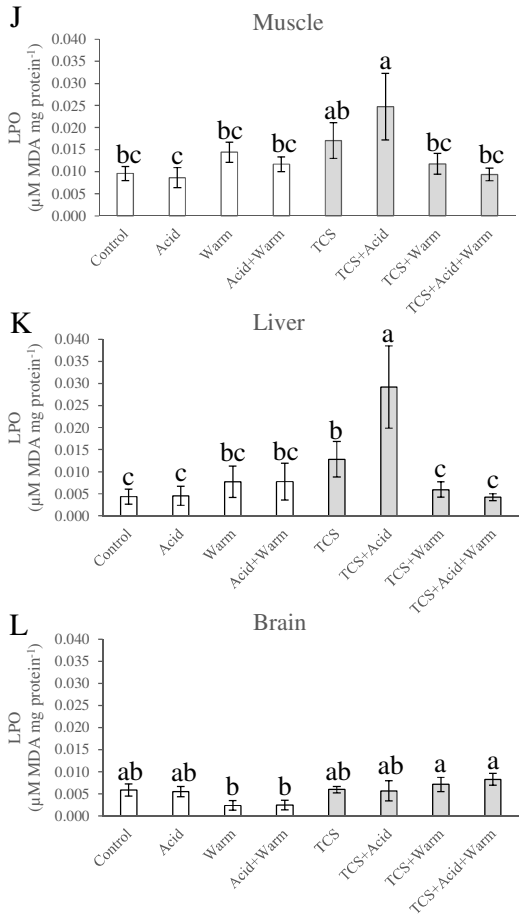


Figure 5.4. ***(continuation)*** Antioxidant activity (CAT, µM min $^{-1}$ mg^{-1} protein; SOD, % inhibition; GST, nmol min $^{-1}$ mg^{-1} protein) and lipid peroxidation (expressed as MDA concentration; µM mg^{-1} protein) in muscle (A, D, G, J), liver (B, E, H, K) and brain (C, F, I, L) of *D. sargus* after 28 days of exposure (mean ± SD; n = 5). Different letters (a-c) indicate significant differences between treatments (for the same tissue; $p < 0.05$). Abbreviations: Control – reference temperature and pH conditions (i.e. T = 19 ºC and pH = 8.0 units); TCS – triclosan exposure; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC), CAT – catalase activity; SOD – superoxide dismutase inhibition; GST – glutathione S-transferase; LPO – lipid peroxidation.

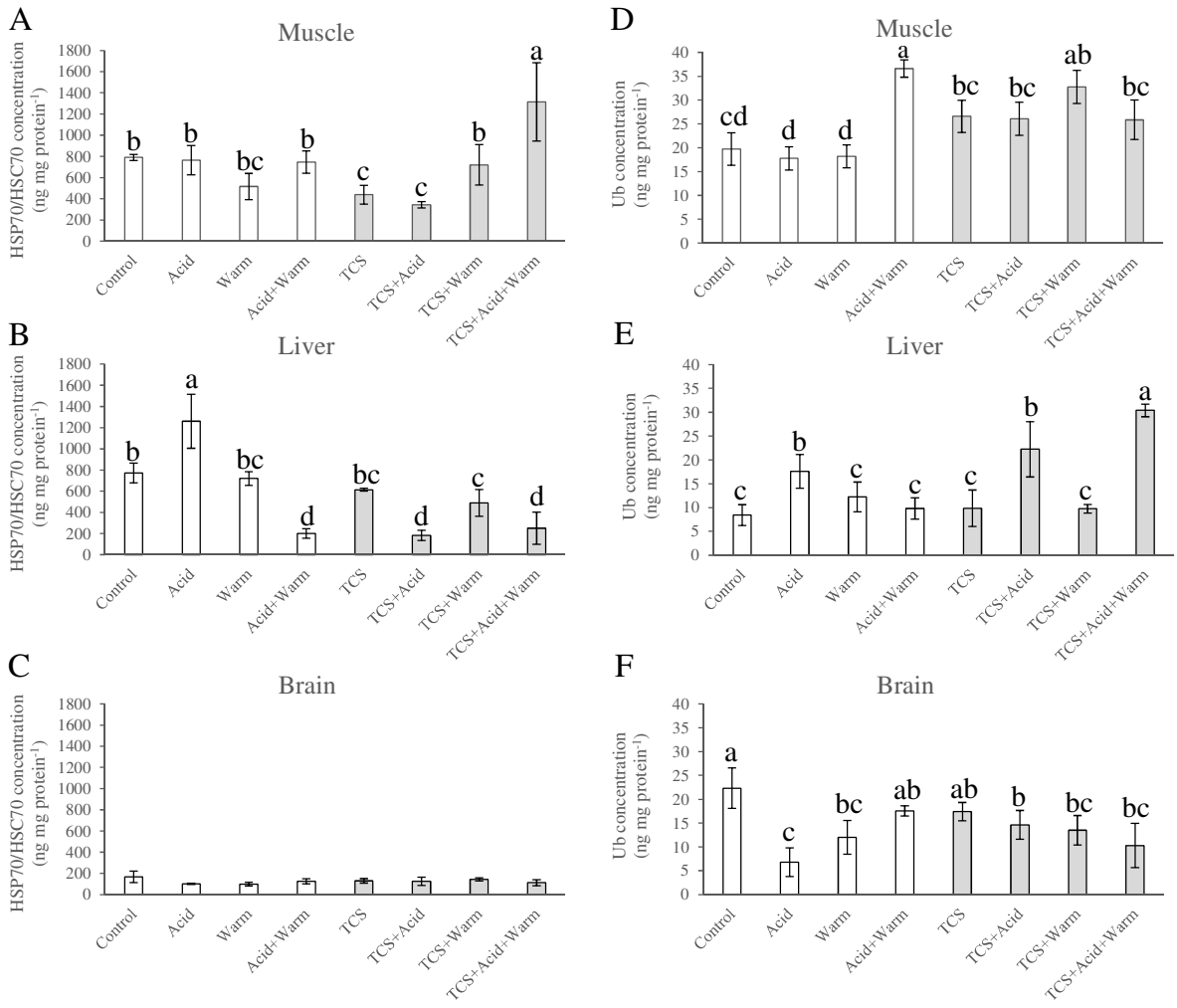


Figure 5.5. HSP/HSC70 (µg mg^{-1} protein) and ubiquitin (Ub; µg mg^{-1} protein) concentrations in muscle (A, D), liver (B, E) and brain (C, F) of *D. sargus* after 28 days of exposure (mean ± SD; $n = 5$). Different letters (a-d) indicate significant differences between treatments (for the same tissue; $p < 0.05$). Abbreviations: Control – reference temperature and pH conditions (i.e. T = 19 ºC and pH = 8.0 units); TCS – triclosan exposure; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC); HSP70/HSC70 – heat shock proteins; Ub – total ubiquitin.

AChE brain activity and VTG liver content are shown in **Figure 5.6**. A significant inhibition of AChE brain activity was observed in Warm, Warm+Acid, TCS and TCS+Acid+Warm treatments (activity decreases between 22.4% and 28.1% in relation to Control treatment; $p < 0.001$ in all cases), while no significant effects were observed in Acid, TCS+Warm and TCS+Acid treaments ($p > 0.050$; **Figure 5.6.A** and **Annex 8, Table A.8.7.**). Furthermore, this AChE activity inhibition was not correlated with TCS brain concentrations, nor with animal fitness indexes (K and BB_{ratio}). As for VTG liver content, the combination of acidification and warming, as well as TCS exposure (regardless of temperature and pCO_2) significantly reduced the production of this protein, with TCS+Acid and TCS+Acid+Warm treatments registering the

highest inhibition in relation to the Control treatment (i.e. a decrease around 80% in relation to Control treatment; $p < 0.001$ in both cases; **Figure 5.6.B** and **Annex 8, Table A.8.7.**). In addition, a significant negative correlation found between VTG content and K ($r = 0.37$ and $p < 0.050$; **Table 5.2.**).

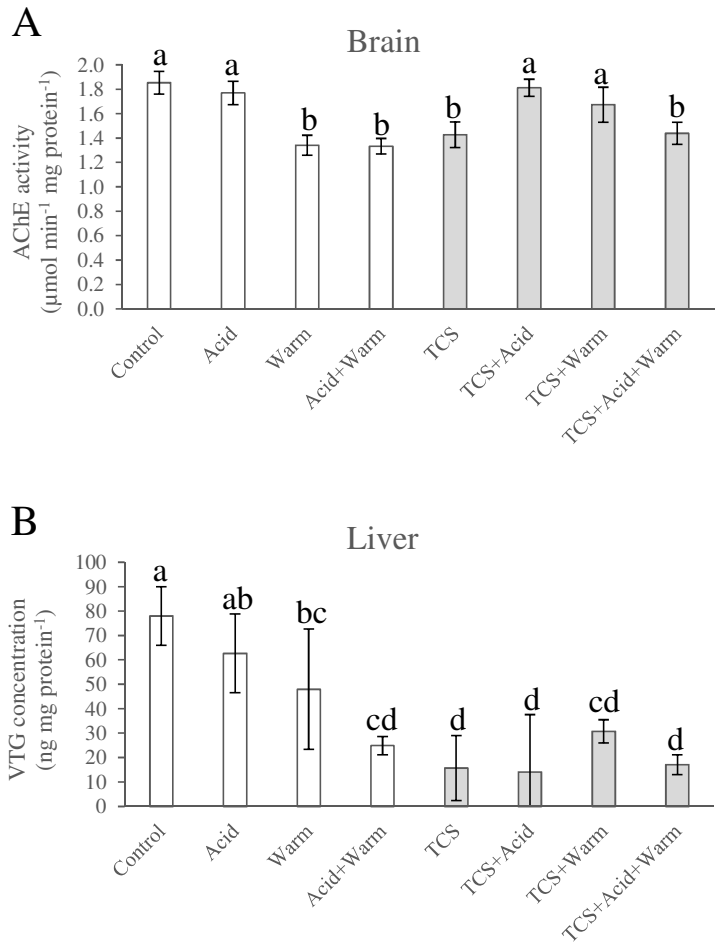


Figure 5.6. Acetylcholinesterase (AChE) activity (µmol min $^{-1}$ mg^{-1} protein; A) in brain tissues and vitellogenin (VTG) concentration (ng mg^{-1} protein; B) in liver tissues of *D. sargus* after 28 days of trial (mean ± SD; $n = 5$). Different letters (a-d) indicate significant differences between treatments ($p < 0.05$). Abbreviations: TCS – triclosan exposure; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC); AChE – acetylcholinesterase activity; VTG – vitellogenin concentration.

4. Discussion

The present study provided relevant and innovative findings showing that TCS bioaccumulation mechanisms in marine fish species are influenced by the surrounding abiotic conditions and that increased temperature and pCO_2 levels can be generally associated with lower TCS tissue burdens. As for the ecotoxicological responses of juvenile *D. sargus*, TCS dietary exposure significantly increased LPO, as well as decreased AChE brain activity and VTG liver

content, but less evident effects were observed in fish antioxidant machinery (CAT, SOD and GST activities). Furthermore, these effects were either exacerbated or attenuated/reversed by the co-exposure to abiotic stressors, with the combination of TCS and acidification resulting, overall, in more notorious biochemical alterations (namely, in SOD, LPO, HSP70/HSC70, Ub and VTG) compared to the other treatments.

4.1. TCS concentration in feeds, seawater and non-contaminated fish samples

TCS was efficiently incorporated in TCS-contaminated feed, evidencing a stable concentration during the whole trial (i.e. TCS in feed did not undergo degradation/loss throughout time). The fact that TCS was not detected in CTR feed indicates that no external contamination occurred during feed preparation. Furthermore, seawater and fish samples from Control treatment also revealed TCS levels below LOD, thus, confirming that during the trial no external contamination has taken place within the independent recirculation aquaculture system, apart from the intended TCS dietary exposure in contaminated treatments. In this way, the quantifiable (and similar among treatments) TCS levels in seawater samples from contaminated treatments after 28 days of dietary exposure to TCS can only be attributed to two reasons: i) TCS partial leaching from contaminated feed to seawater, which seems less likely to have occurred given the physical and chemical properties of this compound (i.e. low water solubility and high adsorption to solid particles; Dhillon et al., 2015); and ii) faecal excretion by fish of TCS in its parental form (Escarrone et al., 2016; Arnot et al., 2017). It should be noted that the low, but not negligible, TCS levels in seawater from contaminated treatments, to which fish were chronically exposed during the trial, might have also contributed (even if to a much lower extent compared to the intended dietary exposure) to the final TCS tissue burden due to its re-uptake through fish gills (i.e. via inhalation), possibly enhancing the ecotoxicological effects elicited by the dietary exposure to this compound (as well as the studied abiotic stressors).

4.2. TCS tissue bioaccumulation and effects of warming and acidification

Even though PPCPs are generally assumed to be mostly uptaken by marine biota through direct contact with contaminated water masses (i.e. via gills through inhalation, or via skin), recent studies have pointed out dietary transfer (i.e. biomagnification along the food webs) as an important pathway for contaminants' bioaccumulation in marine fish species, particularly for organic and/or less hydrosoluble compounds (e.g. Dijstra et al., 2013; Zenker et al., 2014; Maulvault et al., 2016, 2018b). Hence, even though TCS exposure concentration selected in this study may not mimic a dietary exposure that occurs in nature (e.g. TCS levels in bivalve species, which are natural preys of *D. sargus*, are around 2 ng g^{-1} dw, i.e., ~7 times lower than TCS

concentration in the contaminated feed; Álvarez-Muñoz et al., 2015), the present results constitute a proof of concept that, indeed, TCS can be uptaken by marine fish species through diet, with muscle being the primary organ for bioaccumulation of this compound. To date, studies on TCS bioaccumulation in different fish tissues are extremely limited (Escarrone et al., 2016) and, to the best of the authors' knowledge, no such studies have been carried out using marine species exposed to TCS via diet, thus, hindering comparisons of the present results with previous reports. Yet, the lipophilic behaviour of TCS suggests that it can be highly bioaccumulative and persistent in fatty tissues, such as liver and gonads, while much lower bioaccumulation is expected to occur in tissues with lower lipid content, such as muscle and brain (Dhillon et al., 2015; Escarrone et al., 2016). Indeed, Escarrone et al. (2016) detected higher TCS concentrations in fish gonads and liver, and lower levels in gills, brain and muscle after 14 days of TCS water exposure (160 µg L^{-1}). Such findings contradict the pattern observed in the present study (i.e. muscle TCS concentrations were ~10 times higher than those of liver, under the reference temperature and pCO_2 conditions), suggesting that contaminant exposure route, dose and time may play a preponderant role on TCS's mechanisms of bioaccumulation and tissue distribution in fish. On the other hand, the surprisingly lower persistence of TCS in its parental form observed in the liver could be due to an intensive compound biotransformation that might have occurred in this tissue, since liver is the primary organ for the metabolization and detoxification of xenobiotics, being then transported to other organs through the blood circulation or excreted via the hepatobiliary route (Wang et al., 2010; Maulvault et al., 2016). The toxicokinetic mechanisms of TCS in fish species are still poorly understood, however, recent data have shown that TCS is intensively and rapidly metabolized in the liver, where it partially undergoes glucuronidation or sulfonation, being subsequently transformed into more soluble and easily excreted metabolites (James et al., 2012). Such TCS detoxification mechanism seem to have been enhanced by seawater warming and acidification (although, to a lower extent in this last case), substantially reducing TCS levels in all tissues.

Warmer temperatures often enhance fish metabolism, which translate into increased ventilation and feeding rates in order to fulfil the higher metabolic demands (Rosa et al., 2013, 2014; Anacleto et al., 2018). Thus, although an enhanced animal metabolism can result in higher contaminants' uptake (of those dissolved in the water column, i.e. via respiration, or those present in the feed, i.e. via ingestion; e.g. Dijkstra et al., 2013; Hedrick-Hopper et al., 2015; Maulvault et al., 2016, 2018b), it can also facilitate contaminants' metabolization and elimination (Maulvault et al., 2016, 2018b; Serra-Compte et al., 2018). Moreover, increased metabolic rates can enhance animal growth, which can also translate into lower contaminant bioaccumulation due to somatic growth dilution (Dijkstra et al., 2013; Maulvault et al., 2016). This argument may also explain the lower TCS tissue burden in fish exposed to warming conditions, which match the generally higher W and TL also found in these treatments, as well as the negative correlation observed between

these parameters and TCS concentrations in liver and brain of contaminated fish. As follows, here we argue that the enhanced metabolism of *D. sargus* elicited by 28 days of exposure to +5 ºC of seawater temperature could have been accompanied by an increase in both animal growth and TCS metabolization/excretion, therefore, translating into lower TCS tissue burdens in fish under warming. Yet, such pattern seemed to have been somewhat reversed by acidification in fish muscle, as TCS+Acid+Warm treatment showed higher TCS concentrations than TCS+Warm treatment, but similar values to those observed in TCS+Acid treatment.

Contaminants' chemical properties are largely influenced by environmental conditions, with metals and other ionic compounds being particularly affected by the surrounding seawater pH levels. Such is the case of TCS that becomes increasingly protonated and loses its negative charge as pH falls below ~8.0 units (Orvos et al., 2002; Rowett et al., 2016). In this way, a recent study using the freshwater amphipod *Gammarus pulex* as model organism evidenced increased TCS toxicity under lower pH levels (i.e. 7.3 pH units *versus* 8.4 pH units), and such results were justified by the fact that cell lipid membranes are generally impermeable to ionised molecular forms (i.e. ionized TCS forms are less toxic; Rowett et al., 2016). Yet, the present results are not in agreement with this argument, since fish exposed to acidification alone also exhibited lower TCS tissue burdens than those under normal temperature and pCO_2 conditions, though not as much as warming. Such results can be explained by the three following points. First, the study of Rowett et al. (2016) was focused on TCS exposure via water and, therefore, a greater influence of the surrounding pH level on TCS's availability can be expected in this case, in comparison with the conditions simulated in the present study (i.e. TCS exposure via feed). Second, in this previous study, a higher ΔpH was simulated (i.e. a decrease of -0.9 pH units, whereas in the present study ΔpH = -0.4 pH units), as more pronounced changes in pH levels can be expected in freshwater environments likely conditioning the ratio between molecular and ionized TCS forms available for fish uptake to a greater extent than the one elicited in the present study. Third, the energy required to maintain animal homeostasis under -0.4 pH units could have diminished fish biological activities, thus, reducing the uptake rates of TCS. It is also worth mentioning that the trends observed in fish liver and brain under acidification were further enhanced when warming was also added to the equation (i.e. lower values in TCS+Acid+Warm treatment compared to TCS+Acid treatment), and that was certainly related to the increased metabolic rates (and, the corresponding enhanced TCS detoxification) induced by +5 ºC of seawater temperature.

Linking the present findings with environmental and seafood safety, despite results do not evidence increased TCS uptake in fish tissues under warming and/or acidification, the trends presently observed clearly confirm that PPCPs' fish tissue burdens are strongly influenced by seawater abiotic variables, which can alter compounds' bioaccumulation, metabolization and elimination mechanisms. Hence, such findings highlight that the interactive effects of abiotic

variables should not be neglected when estimating the environmental hazards posed by PPCPs, nor when regulating the presence of PPCPs in the environment and seafood species. Furthermore, the current lack of literature on this matter, along with the controversial results reported in the few available studies (Hedrick-Hopper et al., 2015; Escarrone et al., 2016) calls for the need to further investigate the impacts of climate change from an ecotoxicological point of view, considering different contaminant exposure routes, as well as exploring less pronounced to more severe ranges of stressors' effects.

4.3. Interactive ecototoxicological effects of TCS, warming and acidification in *D. sargus*

Although animal condition can gradually decline when fish are exposed to environmental stressors due to additional energetic costs that prevent the allocation of energy resources towards somatic growth (Sandblom et al., 2016; Maulvault et al., 2018a; Anacleto et al., 2018), juvenile *D. sargus* did not exhibit significant differences in K (nor in BB_{ratio}) after 28 days of exposure to the experimental conditions. Therefore, results suggest that fish physiological mechanisms were somewhat able to compensate the stress induced by TCS, +5 ºC and/or +1000 µatm pCO_2 (equivalent to -0.4 pH units) during 28 days of exposure trial. On the other hand, fish exposed to TCS alone or combined with acidification revealed, in overall, higher HSI than the other treatments (though statistical significance was only obtained against TCS+Acid+Warm treatment). These results match the trends observed in TCS tissue concentrations (i.e. higher TCS concentrations in TCS and TCS+Acid treatments, along with the positive correlation found between TCS liver concentration and HIS), and are in agreement with the fact that xenobiotics' exposure usually provokes liver hypertrophy and/or hyperplasia (Diniz et al., 2009; Sadekarparwar and Parikh, 2013; Maulvault et al., 2017). Conversely, the significantly lower HSI observed in fish exposed to the combination of the three stressors (compared to TCS exposure alone or combined with acidification) may be associated with the lower TCS liver concentration elicited in this treatment and/or with cell apoptosis promoted by such severe stress conditions.

In agreement with previous findings (e.g. Canesi et al., 2007; Matozzo et al., 2012; Liang et al., 2013; Maulvault et al., 2017, 2018a), the exposure to the three studied stressors resulted in both up- and down regulations of molecular biomarker responses, according to fish tissue and biomarker. The differential tissue responses (e.g. higher CAT activity in muscle than in liver and brain; and CAT muscle activity induced by TCS alone, whereas CAT liver activity was inhibited by TCS plus acidification) were likely related to the fact that different tissues not only can have distinct baseline levels of molecular biomarkers, but may also respond differently to the presence of stressors (such as, TCS, warming and/or acidification), since each tissue is composed by different cell types (i.e. organs are physiologically and functionally different). Hence, the current findings highlight the importance of assessing fish tissue differential responses in

ecotoxicological studies, as such approach provides a broader view on the impacts of environmental stressors in a whole organism context.

The antioxidant enzymes SOD, CAT and GST play a key role in cells' defence mechanisms against the oxidative stress induced by environmental stressors, i.e.: SOD converts superoxide radicals into hydrogen peroxide and molecular oxygen (O_2) and, then, CAT converts hydrogen peroxide into oxygen and water (Halliwell and Gutteridge, 1985), while GST is a major second phase detoxification enzyme (Sheehan et al., 2001), being also responsible for generating less toxic and more hydrophilic compounds through the conjugation of lipid peroxidase breakdown products with glutathione (GSH; Barata et al., 2005; Park et al., 2017). In this way, previous studies conducted in marine organisms have reported an induction of CAT, SOD and GST activities under increased temperature and pCO_2 levels (e.g. Rosa et al., 2016; Jesus et al., 2018; Maulvault et al., 2017, 2018a), as well as under chemical contaminants' exposure (e.g. Matozzo et al., 2012; Maulvault et al., 2017, 2018a), thus, corroborating the findings obtained with *D. sargus* exposed to warming and/or acidification (increased GST brain activity), TCS (increased CAT muscle activity, as well as a positive correlation between TCS concentration and CAT activity in contaminated fish muscle) and TCS co-exposed with acidification (increased SOD liver and brain activities). In agreement, Park et al. (2017) reported increased SOD and GST activities in the copepod *Tigriopus japonicus* short-term exposed to different concentrations of TCS via water (50 and 100 µg L^{-1}). Similarly, in response to TCS water exposure, Matozzo et al. (2012) found a significant increase in SOD gills activity in *Ruditapes philippinarum*, while no changes were observed in CAT activity. Yet, an opposite effect was also observed in our study, with CAT (liver) and GST (muscle and liver) activities being inhibited by the co-exposure of TCS and warming, though such inhibition seemed to have been somewhat attenuated by acidification. Such pattern suggests that, despite the lower TCS levels in TCS+Warm treatment in the different tissues, the severity of stress induced by the combination of TCS exposure and + 5 ºC must have led cells' antioxidant mechanisms to exhaustion, possibly due to an excessive production of H_2O_2 (Gonzalez-Rey and Bebianno; 2014; Maulvault et al., 2018a). SOD muscle activity was also inhibited in Warm and Acid+Warm treatments, most likely due to ROS-mediated denaturation promoted by increased temperatures resulting in the inactivation of this enzyme (Ferreira et al., 2015), but such effect was reversed by TCS co-exposure. Though literature is extremely scarce in what concerns the interactive effects of PPCPs and abiotic variables on marine species ecotoxicological responses (with no studies being focused on cells' antioxidant activity, to the best of the authors' knowledge), a similar trend was also observed in our recent study performed with *Dicenthrachus labrax*, where the increased SOD inhibition in fish muscle promoted by warming and acidification was partially attenuated by diclofenac co-exposure (Maulvault et al., 2018a).

In line with the enhanced CAT and SOD activities, TCS exposure alone or combined with acidification elicited the formation of lipid peroxides in fish muscle and liver (i.e. increased MDA concentration in relation to the remaining treatments), a result that is most likely related with the higher TCS levels found in fish exposed to these conditions. The increased LPO found in these treatments suggests that the enhancement of antioxidant defences was only able to prevent the oxidative stress induced by TCS and acidification to some extent, leading to the consequent cell damage.

Matching some of the present findings (i.e. HSP70/HSC70 content in fish liver under acidification and muscle of fish exposed to the three stressors alone), the synthesis of molecular chaperones, such as HSPs, can be induced in order to repair, refold, and/or eliminate damaged proteins, thus preventing cellular damage promoted by the exposure to both abiotic stressors (e.g. Rosa et al., 2014; Pimentel et al., 2015; Madeira et al., 2016a,b) and chemical contaminants (e.g. Maulvault et al., 2017, 2018a), including TCS (Lin et al., 2014). The positive correlation found between TCS and HSP70/HSC70 concentrations in this tissue is consistent with this trend, thus evidencing that chaperoning mechanisms were further enhanced as TCS tissue levels increased (and *vice-versa*). Yet, chaperone activation mechanisms cannot be looked at as straightforward process, as they are influenced by several physiological factors, including stress levels, interactions with chemical contaminants, species, tissue and animal hormone levels, (e.g. Mahmood et al., 2014; Madeira et al., 2016b). Furthermore, HSPs can also be down-regulated due to the exhaustion of cytoprotective systems and metabolic depression promoted by severe and/or chronic stress conditions (Madeira et al., 2016b; Maulvault et al., 2018a). Such was the case of fish liver exposed to acidification and/or warming (regardless of TCS exposure), as well as muscle of fish exposed to TCS and acidification. Similarly, a previous study with *D. labrax* reported that HSP70/HSC70 synthesis in fish muscle was up-regulated by acidification and warming in co-exposure with diclofenac, but down-regulated by acidification alone (Maulvault et al., 2018a). Falisse et al. (2017) also reported HSPs overexpression in zebrafish (*Danio rerio*) larvae following TCS seawater exposure at 100 µg L^{-1}, but not at 50 µg L^{-1}, suggesting that TCS mode of action may be dose-dependent.

Similar to HSPs, Ub synthesis can be induced when internal stress is generated, in order to signal denatured proteins to be degraded by the proteasome (e.g. Gravel, and Vijayan, 2007; Madeira et al., 2017). This pattern was observed in fish liver exposed to increased pCO_2 levels (i.e. Acid, Acid+Warm, TCS+Acid and TCS+Acid+Warm treatments), where increased Ub levels were generally negatively correlated with lower K. Furthermore, results also indicate that the synthesis of these proteins was positively correlated with TCS levels in liver and brain. Conversely, extreme or long-lasting stress conditions can also result in Ub inhibition due to physiological collapse, since protein synthesis is an energy demanding process, requiring over 50% of all O_2 consumed my fish (e.g. Gravel, and Vijayan, 2007; Madeira et al., 2016b). Indeed,

the overall decrease Ub content observed in fish brain suggests that the exposure to TCS, acidification and/or warming led to irreversible damages, that both chaperoning and ubiquitin-proteosomal pathways could not compensate for, most likely as a result of a poorer energy budget (and aerobic scope) in fish under these stressors (Falfushynska et al., 2014; Madeira et al., 2017). Moreover, Ub inhibition was particularly evident under acidification alone or combined with TCS and warming, and that may be related to the fact that elevated pCO_2 levels disrupt the ionic balance in proton-based neurotransmitter cell receptors (e.g. $GABA_A$), with the consequent increase of animal stress and cognition impairments (e.g. Nilsson et al., 2012; Hamilton et al., 2014; Munday et al., 2014; Maulvault et al., 2018c).

AChE was also inhibited by warming alone or by the co-exposed with acidification, and by TCS alone or by the co-exposure to the two abiotic stressors simultaneously. These results are in line with previous findings: i) the exposure to abiotic stressors, particularly increased pCO_2 levels, strongly mediates AChE brain activity (Rosa et al., 2016); and ii) AChE can be a target for chemical contaminants, leading to the inhibition of its activity and, therefore, to failed synaptic transmission and muscle overstimulation (Schmidel et al., 2014; Maulvault et al., 2017; Topal et al., 2017). Furthermore, AChE activity inhibition seemed to be consistent with the Ub synthesis impairment in the brain, particularly in TCS-exposed treatments, suggesting that TCS dietary exposure may have a neurotoxic effect in fish species. In accordance with the present results, Hedrick-Hopper et al. (2015) observed impaired reflexes in Atlantic croaker (*Micropogonias undulatus*) following TCS dietary exposure, with the dorsal fin response being further affected by the combination of TCS and increased temperatures, evidencing that TCS's toxicological attributes may be accentuated by the predicted climate change seawater conditions. It is also worth noting that Falisse et al. (2017) reported acute neurotoxicological effects in *D. rerio* larvae exposed to TCS (50 and 100 µg L^{-1}), though such effects translated into an induction of AChE activity rather than an inhibition, contrasting with the present findings.

VTG is usually very low or undetectable in male and juvenile individuals, but can reach high values in the liver and plasma of mature females. Nonetheless, the exposure to endocrine disrupting compounds has been previously described to either elicit its production in males/juveniles (estrogenic effect) or inhibit its production in females (antiestrogenic effect; Matozzo et al., 2008; Gonzalez-Rey and Bebianno, 2014), causing remarkable reproductive impairments and, ultimately, deleterious ecological consequences. Such impacts may be even more severe in protandrous fish, such as those belonging to the *Sparidae* family, which can alternate from one sex to another throughout its life cycle. This is the case of *D. sargus*, which is a digynic hermaphrodite species, i.e. some early life-stage individuals exhibit non-functional bisexual gonads, which can develop into male or female due to various environmental factors (e.g. demographics, social behaviour, abiotic conditions and insufficient nutrition; once mature,

some males retain the ability to invert sex into secondary females (Morato et al., 2003). Moreover, since marine fish species' reproductive strategies are strongly mediated by the surrounding abiotic conditions, warming and acidification are also expected to affect their reproduction and ecological success, or even to accentuate their negative responses to the presence of endocrine disrupting compounds (e.g. Morato et al., 2003; Pankhurst and Munday, 2011; Cripps et al., 2014). These arguments are in accordance with the present findings, since TCS dietary exposure drastically inhibited VTG synthesis in relation to the values observed under control conditions and, therefore, showed a potential antiestrogenic effect in juvenile *D. sargus*, as did warming and acidification (except when these last two acted alone). Interestingly, the greatest inhibition was observed under the combination of TCS and increased pCO_2 levels (i.e. TCS+Acid and TCS+Acid+Warm treatments). These results follow the trend observed in an earlier study with *D. labrax*, i.e. acidification and/or warming also seemed to have an antiestrogenic effect (i.e. lowering VTG levels) by counteracting the effects of dietary exposure to diclofenac (Maulvault et al., 2018a). Even though the effects of increased temperatures are, by far, better described in literature (e.g. Chandra et al., 2012; Hedrick-Hopper et al., 2015; Maulvault et al., 2018a), previous studies also pointed out that acidification can play a preponderant role on marine biota's endocrine regulation (e.g. by modulating the synthesis of cortisol levels and thyroid hormones, which are, in turn, linked to VTG induction; McCormick and Bradshaw, 2006; Kwong et al., 2014). Yet, little is still known regarding interactive effects of co-exposure to acidification and endocrine disruptors, thus calling for the need to further investigate the effects of environmental stressors on fish endocrine system and reproduction.

5. Conclusions

Data confirmed that TCS uptake and elimination mechanisms in the marine fish species *D. sargus* are strongly influenced by seawater temperature and pCO_2 levels, and that the metabolic and physiological changes elicited by warmer and/or more acid environmental conditions can result in lower TCS tissue burdens.

As for ecotoxicological responses, results showed that TCS dietary exposure promoted cellular damage (increased LPO), neurotoxicity (AChE inhibition) and endocrine disruption (VTG inhibition) in juvenile *D. sargus,* while its effect on tissues' antioxidant machinery (CAT, SOD and GST activities) was not so clear. Yet, the differential responses (up- or down-regulation) observed according to tissue pointed out that TCS can have distinct modes of action depending on animal tissue and enzyme. TCS toxicological attributes were also largely influenced by increased temperature (+5 ºC) and pCO_2 levels (+1000 µatm), resulting in an enhancement or attenuation of biochemical biomarker responses. Overall, TCS co-exposed with acidification resulted in more drastic biochemical alterations than those elicited by TCS co-exposed with

warming or even the combination of the three stressors, resulting in increased SOD activity and LPO, as well as in diminished chaperoning and VTG synthesis. Hence, the distinct effects promoted by TCS exposure, acidification and warming, acting alone or combined, highlight the need to consider the interactions between environmental stressors in future ecotoxicological studies, as well as to incorporate these variables in future regulations and recommendations regarding the environmental presence of PPCPs. Such actions will be crucial to understand and mitigate the toxicological implications of climate change, as environmental stressors unlikely occur in isolation, nor the alterations of environmental conditions affect marine ecosystems in the same way across the planet.

Ethical statement

Fish trials were performed according to European regulations (EU Directive 2010/63), and previously approved by the Ethical Committee of the Faculty of Sciences of the Lisbon University. All researchers involved in fish maintenance, handling and sampling were previously accredited by the Federation of European Laboratory Animal Science Associations (FELASA).

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CHAPTER 6.

ECCs MIXTURES: TOXIC METALS (iAs), FLAME RETARDANTS (DEC 602, DEC 603, DEC 604 AND TBBPA) AND PERFLUORINATED COMPOUNDS (PFOS AND PFOA)

Assessing the effects of seawater temperature and pH on the bioaccumulation of emerging chemical contaminants in marine bivalves

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Abstract

Emerging chemical contaminants [e.g. toxic metals speciation, flame retardants (FRs) and perfluorinated compounds (PFCs), among others], that have not been historically recognized as pollutants nor their toxicological hazards, are increasingly more present in the marine environment. Furthermore, the effects of environmental conditions (e.g. temperature and pH) on bioaccumulation and elimination mechanisms of these emerging contaminants in marine biota have been poorly studied until now. In this context, the aim of this study was to assess, for the first time, the effect of warmer seawater temperatures (Δ = +4 ºC) and lower pH levels (Δ = -0.4 pH units), acting alone or combined, on the bioaccumulation and elimination of emerging FRs (dechloranes 602, 603 and 604, and TBBPA), inorganic arsenic (iAs), and PFCs (PFOA and PFOS) in two estuarine bivalve species (*Mytilus galloprovincialis* and *Ruditapes philippinarum*). Overall, results showed that warming alone or combined with acidification promoted the bioaccumulation of some compounds (i.e. dechloranes 602, 604, TBBPA), but also facilitated the elimination of others (i.e. iAs, TBBPA). Similarly, lower pH also resulted in higher levels of dechloranes, as well as enhanced iAs, PFOA and PFOS elimination. Data also suggests that, when both abiotic stressors are combined, bivalves' capacity to accumulate contaminants may be time-dependent, considering the significantly drastic increase observed with Dec 602 and TBBPA, during the last 10 days of exposure, when compared to reference conditions. Such changes in contaminants' bioaccumulation/elimination patterns also suggest a potential increase of human health risks of some compounds, if the climate continues changing as forecasted. Therefore, this first study pointed out the urgent need for further research on the effects of abiotic conditions on emerging contaminants kinetics, to adequately estimate the potential toxicological hazards associated to these compounds and develop recommendations/regulations for their presence in seafood, considering the prevailing environmental conditions expected in tomorrow's ocean.

Keywords: emerging chemical contaminants, flame retardants, toxic elements, perfluorinated compounds, bioaccumulation, warming, acidification.

1. Introduction

The remarkable increase of the human footprint on the planet (i.e. world population constant growth, excessive use of natural resources, and massive production of pollutants), especially since the mid-20th century, has contributed to one of the greatest environmental concerns of our time: climate change. Climate change effects can already be felt in many regions of the world, and are expected to worsen in the coming 50-100 years, with devastating consequences at ecological and human scales, even if strong efforts are made to maintain greenhouse gas emissions (GHGs) at the current levels (IPCC, 2014). Such changes also affect marine ecosystems, leading to a potential increase of the average seawater surface temperature (up to +4 ºC), as well as to ocean acidification due to increased levels of atmospheric CO_2, which dissolves into the ocean, dropping seawater pH down to 0.4 units in some areas of the globe (according to scenario 8.5 of the Representative Concentration Pathways of GHG concentrations, i.e. RCP 8.5, of the Intergovernmental Panel for Climate Change, IPCC, 2014). Depending on the region, both climate change effects can act independently as a single stressor, promoting deleterious alterations in marine species metabolism, growth, reproduction, among others, or can occur simultaneously (i.e. combined with each other or with other climate stressors, e.g. hypoxia, salinity) representing additional challenges to the resilience of marine ecosystems (e.g. Rosa et al., 2016; Maulvault et al., 2016, 2017; Sampaio et al., 2018).

Marine species are currently chronically surrounded by an array of chemical contaminants, particularly those inhabiting areas that are more vulnerable to anthropogenic impacts (e.g. estuaries and coastal lagoons) (e.g. Bollman et al., 2012; Maulvault et al., 2015). New chemical substances that have not been historically recognized as pollutants, and for which limited toxicological information is still currently available, are increasingly more present in the marine environment (e.g. Feo et al., 2012; Cunha et al., 2015; Marques et al., 2015; Vandermeersch et al., 2015). These "contaminants of emerging concern" include various compounds from distinct chemical groups, which may occur naturally in the environment [e.g. inorganic arsenic (iAs)] or are exclusively man-made substances [e.g. flame retardants (FRs), perfluorinated compounds (PFCs), pharmaceutical residues, UV-filters and musks, among others] commonly derived from domestic, hospital and industrial effluents, as well as, agriculture and aquaculture activities (e.g. Bollman et al., 2012; Feo et al., 2012; Maulvault et al., 2015; Vandermeersch et al., 2015). Arsenic is an ubiquitous element that can occur in the aquatic environment in several oxidation states (-3, 0, +3 and +5), although being mostly found in its most toxic forms, i.e. the inorganic ones (AsIII or AsV; Matschullat, 2000). Arsenic concentrations in coastal ecosystems may range from 1 to 20 µg L^{-1} (Smedley and Kinniburgh, 2001), and in biota the highest concentrations are usually found in bivalve species (total As concentrations up to 24 µg g^{-1} dry weight (dw) (Sloth et al., 2008; Maulvault et al., 2015). Flame retardants (FRs) are persistent contaminants in the

environment, particularly accumulating in sediments and biota, since most of them are extremely hydrophobic. Thus, their concentrations in seawater are usually undetectable or within the range of pg L^{-1} (e.g. Bollmann et al., 2012), whereas in marine sediments and biota values are in the order of pg g^{-1} dw and ng g^{-1} lipid weight (lw), respectively (e.g. Feo et al., 2012; Santín et al., 2013; Vandermeersch et al., 2015). Tetrabromobisphenol A (TBBPA) is one of the most relevant FR widely used by the industry and can be found in river and estuarine sediments, as well as in biota, reaching up to 14 ng g^{-1} of lw (EFSA, 2011; Vandermeersch et al., 2015). As for other types of FRs, based on their known toxic effects, recently, the EU banned or restricted the use of certain compounds (e.g. PBDEs, Mirex), which were replaced by new substances, defined as emerging FRs [e.g. dechloranes (Decs); Feo et al., 2012] for which limited information is available about their levels in marine environments. PFCs (e.g. perfluorooctanesulfonic acid, PFOS, and perfluorooctanoic acid, PFOA) are molecules composed by carbon chains strongly bound to fluorine atoms, which are widely used in various industrial and consumer activities (e.g. stain-resistant coatings for fabrics and carpets, fire-fighting foams and floor polishes, among others). Their strong carbon:fluorine bounds make them extremely resistant to degradation and, therefore, persistent over time in biological compartments. In aquatic environments, PFCs' concentrations can vary from undetectable to values surpassing 100 ng L^{-1} (Flores et al., 2013) and even exceeding 500 ng g^{-1} of wet weight (ww) in aquatic biota (e.g. EFSA, 2008; Vandermeersch et al., 2015).

Chemical contaminants' availability in marine sediments/water column and toxicity to biota are strongly influenced by environmental drivers, such as temperature, pH, salinity, upwelling and stratification events. Recent literature has intensively described climate change to likely have a direct impact on contaminants' physical-chemical properties and their partitioning among biological compartments (e.g. Noyes et al., 2009; Marques et al., 2010). Moreover, by altering species physiological status and, at the same time, exacerbating many forms of water pollution, climate change can promote deleterious impacts on marine organisms' physiology, hindering them to cope with the presence of chemical contaminants in the same way as they did before (e.g. Marques et al., 2010; Maulvault et al., 2016, 2017; Sampaio et al., 2018). On the other hand, changes in contaminants' uptake, retention and detoxification rates due to climate change may certainly compromise seafood safety, thus rising concerns from the public health point of view (e.g. Marques et al., 2010; Maulvault et al., 2016). Yet, given the limited number of empirical studies, with most available information being based on mechanistic approaches, the interaction between climate change and pollution still requires further understanding.

In this context, the aim of this study was to assess, for the first time, the effect of temperature (Δ = +4 ºC) and pH (Δ = -0.4 pH units), acting alone or in combination, on the bioaccumulation and elimination of emerging chemical contaminants (Dec 602, Dec 603, Dec 604, iAs, TBBPA, PFOA and PFOS), using estuarine bivalve species (*Mytilus galloprovincialis* and *Ruditapes*

philippinarum) as biological models. The selection of the model species was based on the fact that these species can be considered suitable bioindicators of environmental pollution, since: i) they are filter-feeding and benthic organisms, thus potentially accumulating high levels of chemical contaminants, either dissolved or in suspended particulate forms, often reaching concentrations several orders of magnitude above the surrounding levels (e.g. Maulvault et al., 2015); and ii) they are among the most commercially important seafood species (both wild or farmed), with high consumption frequencies in Europe, thus enabling to establish a link with seafood safety.

2. Materials and Methods

2.1. Experimental design

2.1.1. Chemical contaminants

The contaminants used in each exposure trial were purchased from Toronto Research Chemical Inc. (Toronto, ON, Canada) in the case of Dec 602 ($C_{14}H_4Cl_{12}O$, ≥ 98%), Dec 603 ($C_{17}H_8Cl_{12}$, ≥ 98%) and Dec 604 ($C_{13}H_4Br_4Cl_6$, ≥ 98%), or Sigma-Aldrich (USA) in the case of TBBPA ($(CH_3)_2C[C_6H_2(Br)_2OH]_2$) ,97%), iAs ($H_3AsO_4$, i.e. As(v) oxide, >95%), PFOS (heptadecafluorooctanesulfonic acid solution, $CF_3(CF_2)_7SO_3H$, 10 µg mL^{-1}) and PFOA ($CF_3(CF_2)_6COOH$, 96%). The remaining reagents used to perform the quantification of each contaminant were of analytical grade or higher, and are given in **section 2.2. Quantification of emerging contaminants** throughout the description of the respective methodologies, as well as in **Annex 9, Materials and Methods**.

2.1.2. Contaminant exposure

To perform contaminant exposure, two different approaches (i.e. exposure routes) were considered taking into account the specific physical-chemical properties of each selected compound: i) Trial I - Exposure via dietary sources through contaminant enriched feed. This exposure was carried out for compounds with hydrophobic behaviour, that are more commonly detected in marine sediments or can potentially be biomagnified along the food chain (due to their long half-lives in animal tissues). Thus, the compounds tested were Dec 602 (water solubility (WS) = 8.49 x 1^{0-3} mg mL^{-1}), Dec 603 (WS = 3 x 1^{0-2} mg mL^{-1}), Dec 604 (WS = 21 x 1^{0-3} mg mL^{-1}; Feo et al., 2012) and TBBPA (WS = 6.3 x 1^{0-5} mg mL^{-1}; EU, 2012); and ii) Trial II - Exposure via seawater spiked with contaminant solution. This exposure was carried out for compounds with high water solubility and which are commonly detected in seawater samples. Thus, the compounds tested were iAs (WS > 12 mg mL^{-1}; US National Research Council, 1977), PFOA (WS = 3.4 mg mL^{-1}; EFSA, 2008) and PFOS (WS = 0.52 mg mL^{-1}; EFSA, 2008).

As shown in **Table 6.1.**, TBBPA was not successfully incorporated, using the available feed production methodology nor detected in the experimental feed bioaccumulation. Thus, since TBBPA bioaccumulation could not be studied using the first experimental approach (exposure via contaminant enriched feed), the second experimental approach (i.e. exposure via contaminated seawater) was used instead to study this compound.

For the first approach (i.e. exposure via contaminant enriched feed), bivalve powder feeds (particle size ~40 µm) were manufactured by SPAROS Lda (Olhão, Portugal). Briefly, ingredients were blended in a paddle mixer (Mainca RM90, Spain), micropulverized in a hammer mill (Hosokawa Micron, SH1, The Netherlands) and sieved below 25 micron. This feed was composed by 72.4% crude protein and 12.1% crude fat (full composition of feed can be consulted in **Annex 9, Table A.9.1.**). Contaminant stock solutions were then prepared, by solubilising an amount of each contaminant in < 5 mL chloroform (96%, Merck, USA), in order to achieve the nominal concentrations presented in **Table 6.1.** (i.e. ~100x the average concentrations found in marine sediments of contaminated coastal areas, to assure a clear bioaccumulation/elimination pattern was obtained during the selected experimental time; e.g. Feo et al., 2012; Sühring et al., 2015). Contaminant stock solutions were diluted in deionized water (total volume of 25 mL) and these solutions were top-sprayed on batches of the powder feeds with a pressurized spraying container (standard flat-fan nozzle; size 10 micron; pressure 2.7 bar). Upon coating the contaminant enriched feeds were dried in a convection oven (OP 750-UF, LTE Scientifics, United Kingdom) for 20 min at 40 ºC. After feed preparation, contaminants' final concentration was then determined according to the methodologies described below (see **section 2.2. Quantification of emerging contaminants**; **Table 6.1.**). A non-contaminated feed (maintenance feed to be used during the elimination phase of Trial I and during the whole Trial II) with the same composition was also prepared, following the same preparation protocol previously described, but without including the contaminant stock solutions, to maintain bivalves from control treatments (CTR) in both trials (I and II).

For the second approach (i.e. exposure via contaminated seawater; Trial II), contaminants stock solutions were prepared, by first solubilising an amount of each contaminant in < 5 mL of solvent (methanol, chloroform, or acid nitric, according to contaminant's chemical properties). All solvents were purchased from Merck (USA), and were of liquid chromatography gradient grade (methanol and chloroform) or supra pure (nitric acid 65%). The final volume of each stock solution was then adjusted with seawater to 500 mL (target nominal concentration shown in **Table 6.1.**; nominal concentrations of ~10x the mean concentrations found in seawater samples from contaminated coastal areas were prepared, except for TBBPA for which 10 µg L^{-1} were used, to assure a clear bioaccumulation/elimination pattern was obtained during the experiment; e.g. Smedley and Kinniburgh, 2001, Flores et al., 2013).

2.1.3. Animal collection and acclimation

Bivalve specimens (Trial I: Japanese carpet shell clam, *Ruditapes philippinarum*, *n* = 1100, collection date – May 2015; Trial II: Mediterranean mussel, *Mytilus galloprovincialis*, *n* = 1100, collection date – March 2016) with similar dimensions were collected from the Tagus estuary (Lisbon, Portugal) and transported in appropriate refrigerated boxes (4 ºC) to Guia Marine Laboratory (Faculty of Sciences, University of Lisbon, Portugal). Once at the laboratory, bivalves were randomly and equitably distributed in different rectangular shaped glass tanks (70 L of total capacity; 3 tanks per treatment) of a recirculation aquaculture system (RAS). Each tank had independent functioning. Temperature was set and adjusted whenever needed using an automatic seawater refrigeration system (± 0.1 °C; Frimar, Fernando Ribeiro Lda, Portugal), as well as, submerged digital thermostats (200W, V2Therm, TMCIberia, Portugal). Seawater pH was set and maintained thanks to individual pH probes (GHL, Germany) connected to a computerized pH control system (± 0.1 pH units; Profilux 3.1N, GHL, Germany), which monitored seawater pH in each tank every 2 s, and adjusted them whenever needed, via submerged air stones, by injecting CO_2 (Air Liquide, Portugal; to decrease pH) or by CO_2-filtered aeration (to increase pH) using air pumps (Stella 200, Aqua One Pro, Aqua Pacific UK Ltd, United Kingdom). Furthermore, each tank was equipped with independent UV disinfection (Vecton 300, TMC Iberia, Portugal) and biological filtration (FSBF 1500, TMC Iberia, Portugal) to maintain seawater quality parameters.

Bivalves were acclimated for a time period of 7 days, at the following conditions: dissolved oxygen > 5 mg L^{-1}; temperature = 19 ± 0.5 °C, pH = 8.00 ± 0.05 units, salinity = 35 ± 1 ‰ and photoperiod of 12 hours light and 12 hours dark (12L:12D). Ammonia (NH_3/NH^{4+}), nitrite (NO^{2-}) and nitrate (NO^{3-}) concentrations were daily checked (Tropic Marin, USA), and kept below detectable levels (i.e. < 0.02 mg L^{-1}), with the exception of nitrates, which were kept below 2.0 mg L^{-1}. Seawater total alkalinity was also measured in every tank on a weekly basis, following a protocol previously described (Sarazin et al., 1999) and the combination of total alkalinity (AT) and pH was used to calculate carbonate system parameters (average values obtained for each treatment can be consulted in **Annex 9, Table A.9.2.**). Bivalves were fed at least three times a day with non-contaminated feed (maintenance diet; 2% of the average animal body weight, bw). On a daily basis, animal condition was checked (i.e. dead animals were removed) and 25% of the total water volume in each incubation tank was exchanged.

Four days before initiating contaminants' exposure, seawater temperature and pH were slowly adjusted (+1 ºC and -0.1 pH units per day) in the corresponding tanks, until reaching the target values in tanks/treatments with higher seawater temperature and/or lower pH. The experimental setup (i.e. crossed treatments) used in both trials is schematized in **Figure 6.1.**, and comprised 5 treatments: i) CTR [(control; non-contaminated treatment to investigate possible external sources of contamination apart from feed (Trial I) or seawater (Trial II), in which specimens were

maintained at reference temperature and pH conditions set according to the average values observed in Tagus estuary during summer (i.e. 19 ºC and 8.0 pH units; Anacleto et al., 2014)], ii) CONT (contaminated and reference temperature and pH conditions]; iii) CONT+Acid (contaminated, reference temperature and pH set at 7.6 units, i.e. ΔpH = -0.4 units according to scenario RCP8.5 of the IPCC, 2014); iv) CONT+Warm (contaminated, reference pH and temperature set at 23 ºC, i.e. ΔTemperature = +4 ºC, according to scenario RCP8.5 of the IPCC, 2014); and v) CONT+Acid+Warm (contaminated, pH set at 7.6 units and temperature set at 23 ºC). Each treatment was composed by three replicates (**Figure 6.1.**).

Figure 6.1. Experimental setup in Trials I and II. Abbreviations: Rep – replicate of each treatment; CTR – control (non-contaminated), 19 ºC, 8.0; CONT - contaminated, 19 ºC, 8.0 pH units; CONT+Acid - contaminated, 19 ºC, 7.6 pH units; CONT+Warm - contaminated, 23 ºC, 8.0 pH units; CONT+Acid+Warm - contaminated, 23 ºC, 7.6 pH units.

2.1.4. Contaminant exposure and elimination (Trials I and II)

In Trial I (i.e. exposure via contaminant enriched feed), *R. philippinarum* from each treatment were daily fed with the respective feeds (contaminated for treatments CONT and non-contaminated for treatment CTR; feed amount = 2% of the average animal bw, divided in at least 3 times a day) for a period of 20 days. Afterwards, an elimination phase was carried out for a similar period of time (i.e. another 20 days) by daily feeding animals from all treatments with non-contaminated feed.

In Trial II (i.e. exposure via contaminated seawater), each contaminated treatment was spiked at day 1 with the contaminant stock solutions in order to achieve the nominal concentrations targeted for each contaminant (**Table 6.1.**), while *M. galloprovincialis* were daily fed with non-

contaminated feed during the 20 days of exposure to contaminants (i.e. control feed; feed amount = 2% of the average animals body weight, bw, divided in at least 3 times a day). The target nominal concentrations, as well as, the final contaminant concentrations in each contaminated treatment are shown in **Table 6.1**. Control tanks (i.e. treatment CTR) were also spiked with equivalent amounts of solvent to ensure that no carrier solvent toxicity occurred. On a daily basis, seawater in each tank was partially replaced (~25%) and a volume of the contaminant stock solution (proportional to the 25% water replacement, thus, accounting for concentration lowering due to water exchange) in order to assure a steady contaminant concentration throughout the exposure phase. Afterwards, an elimination phase was carried out (daily contaminant spiking stopped) for 20 days.

In both trials, seawater physical-chemical parameters were daily checked and adjusted at optimum levels whenever needed as previously described. No mortality was observed during experimental trials I and II. Thirty animals (n = 10 per replicate treatment) were randomly sampled from each treatment on days 0, 2, 10, 20 (exposure), 22, 30 and 40 (elimination). Bivalves' biometric data, i.e. total length (L, cm), height (H, cm), width (WI, cm) of the shell, total weight (W, g) and edible weight (EW, g), were registered. Then, edible tissues were collected, pooled (i.e. n = 3 pools per treatment, per sampling day), immediately frozen at -80 °C (for 24 h), freeze-dried at −50 °C, 10^{-1} atm of vacuum pressure, for 48 h (Power Dry LL3000, Heto, Czech Republic) and kept at -80 °C until contaminant quantification was performed. Seawater samples were also collected from each tank at each sampling day and kept at 4 ºC until further analysis, in order to: i) investigate possible external sources of contamination (seawater from CTR treatments; Trials I and II) ii) assure that no contaminant leaching was occurring from the feed (Trial I); and iii) determine contaminants' final concentrations in seawater of each tank/replicate throughout the 40 days of trial (Trial II).

Table 6.1. Contaminant concentrations (mean ± standard deviation) in contaminated (exposure of Trial I) and non-contaminated (exposure of Trial II and elimination phase of Trials I and II) feeds, seawater (Trials I and II) and bivalve samples from CTR treatment (Trials I and II), as well as certified reference material values and limits of the detection (LOD) and quantification (LOQ) in each methodology used to quantify contaminants. Abbreviations: CTR – control (non-contaminated), 19 ºC, 8.0 pH units; CONT - contaminated, 19 ºC, 8.0 pH units; CONT+Acid - contaminated, 19 ºC, 7.6 pH units; CONT+Warm - contaminated, 23 ºC, 8.0 pH units; CONT+Acid+Warm - contaminated, 23 ºC, 7.6 pH units; LW – lipid weight.

Contaminant	**Nominal concentration (exposure)**	Sampling day	**Final concentration in seawater (µg L^{-1})**					**Final concentration in enriched feed**	**Contaminant concentration CTR bivalves**	**LOD**		**LOQ**	
			CTR	CONT	CONT+Acid	CONT+Warm	CONT+Acid+ Warm			Seawater samples	Bivalve samples	Seawater samples	Bivalve samples
Dec 602	50 ng g^{-1} (Feed)	Days 0-40	< LOD	< LOD	< LOD	< LOD	< LOD	10.0 ± 0.4 ng g^{-1}	< LOD (Trial I)	0.60 µg L^{-1}	21 pg g^{-1} lw	2 µg L^{-1}	70 pg g^{-1} lw
Dec 603	50 ng g^{-1} (Feed)	Days 0-40	< LOD	< LOD	< LOD	< LOD	< LOD	50.0 ± 2.0 ng g^{-1}	< LOD (Trial I)	0.10 µg L^{-1}	7 pg g^{-1} lw	0.3 µg L^{-1}	24 pg g^{-1} lw
Dec 604	50 ng g^{-1} (Feed)	Days 0-40	< LOD	< LOD	< LOD	< LOD	< LOD	1.0 ± 0.1 ng g^{-1}	< LOD (Trial I)	0.10 µg L^{-1}	7 pg g^{-1} lw	0.3 µg L^{-1}	24 pg g^{-1} lw
TBBPA	10 µg L^{-1} (Seawater)	Exposure (Days 2-20)	< LOD	9.6 ± 1.5	4.0 ± 1.3	9.6 ± 1.1	2.0 ± 1.1	-	< LOD (Trial II)	0.10 µg L^{-1}	0.3 µg kg^{-1}	1.0 µg kg^{-1}	0.25 µg kg^{-1}
		Elimination (Days 22-40)	< LOD	0.3 ± 0.5	< LOD	< LOD	< LOD						
iAs	100 µg L^{-1} (Seawater)	Exposure (Days 2-20)	74.6 ± 3.3	138.8 ± 9.3	145.1 ± 7.3	152.8 ± 13.6	145.6 ± 7.3	-	10.9 ± 4.3 ng g^{-1} (Trial II)	0.30 µg L^{-1}	2.0 µg kg^{-1}	1.0 µg L^{-1}	4.0 µg kg^{-1}
		Elimination (Days 22-40)	71.2 ± 2.4	69.2 ± 6.7	75.1 ± 2.3	75.8 ± 4.6	72.3 ± 5.7						
PFOA	1 µg L^{-1} (Seawater)	Exposure (Days 2-20)	< LOD	1.0 ± 0.0	0.8 ± 0.3	1.1 ± 0.2	0.9 ± 0.2	-	< LOD (Trial II)	0.03 µg L^{-1}	0.30 µg L^{-1}	0.05 µg kg^{-1}	0.50 µg kg^{-1}
		Elimination (Days 22-40)	< LOD	< LOD	< LOD	< LOD	< LOD						
PFOA	1 µg L^{-1} (Seawater)	Exposure (Days 2-20)	< LOD	1.0 ± 0.0	1.8 ± 0.3	1.0 ± 0.1	1.1 ± 0.2	-	< LOD (Trial II)	0.03 µg L^{-1}	0.30 µg L^{-1}	0.05 µg kg^{-1}	0.50 µg kg^{-1}
		Elimination (Days 22-40)	< LOD	< LOD	< LOD	< LOD	< LOD						

2.2. Quantification of emerging contaminants

2.2.1. Dechloranes 602, 603 and 604 (Decs)

Dechloranes were extracted from feed and bivalve samples using a previously optimized method (de la Cal et al., 2003, Labandeira et al., 2007). For the dechloranes extraction in seawater, samples were first spiked with an internal standard (13C-syn-DP), followed by an ultrasound assisted extraction using 2 ml of hexane (15 min sonication) and centrifugation (7 min, 3500 rpm). The hexane was transferred to a vial. The extract was then reconstituted with 40 µl of toluene for the instrumental analysis. Dechloranes' quantification in all samples was performed using an Agilent 7890A gas chromatograph coupled to an Agilent 7000B triple quadrupole mass spectrometer (Santa Clara, USA). The instrumental conditions and elution program were based on previous work (Barón et al., 2012). Further details regarding this methodology can be consulted in **Annex 9, Materials and Methods**.

2.2.2. Inorganic arsenic (iAs) and total arsenic (total As)

In seawater samples the total concentration of As was determined, whereas only the inorganic fraction was quantified in bivalve samples (i.e. iAs). The extraction of iAs in bivalve samples was performed as previously described in Rasmussen et al. (2012), and iAs was subsequently quantified following the standard method (EN 16802:2016) recently issued by European Committee for Standardization (CEN, 2016). As species were separated on anion exchange High Performance Liquid Chromatography (1100 HPLC Agilent Technologies, Waldbronn, Germany) and detected by inductively coupled plasma mass spectrometry (Agilent 7500ce ICP-MS, Santa Clara, USA) in no gas mode (As75). Total arsenic in seawater samples was determined by direct injection of samples diluted in nitric acid (2%, v/v) in ICP-MS in no gas mode (As75) with rhodium (Rh103) as internal standard. Further details on these two methodologies can be consulted in **Annex 9, Materials and Methods**.

2.2.3. Tretabromobisphenol A (TBBPA)

TBBPA in bivalve and feed samples was extracted using the following procedure based on QuEChERS – LLE extraction, which was previously described in more detail (Cunha et al., 2017). Sample extracts were analyzed by LC-MS/MS, i.e. a high-performance liquid chromatography (HPLC) system Waters Alliance 2695 (Waters, Milford, MA, USA) interfaced to a Quattro Micro triple quadrupole mass spectrometer (Waters, Manchester, UK). TBBPA was analysed in seawater by direct injection on LC-MS/MS with prior addition of 80 µL of $TBBPA_{13}C_{12}$ (IS, 1000 µg L^{-1}). The optimized MS/MS parameters for the target analytes are described in Cunha et al.

(2017). Further details regarding this methodology can be consulted in **Annex 9, Materials and Methods**.

2.2.4. Perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS)

PFOS and PFOA were extracted and analysed in both seawater and biota samples using the methods previously described in Kwadijk et al. (2010). Instrumental analysis was performed using a Thermo Finnigan (Waltham, MA) Surveyor Autosampler and an HPLC system coupled with a Thermo Finnigan LCQ advantage Ion-Trap MS instrument with electrospray (ESI-MS/MS). Further details regarding this methodology can be consulted in **Annex 9, Materials and Methods**.

2.3. Data analysis

Animal condition index (CI_t) was calculated, in ww basis, according to Maguire et al. (1999):

$$CI_t = \frac{W_t}{L_t \text{ x } H_t \text{ x } WI_t} \text{ x } 10{,}000$$

where, *t* is the sampling time in days, W is the animal total weight (g), and L, H and W are the length, height and width of the shell (cm), respectively. Bivalves' growth rate (GR; mg of ww day^{-1}) was calculated as described by Santana et al. (2017):

$$GR_t = (W_t - W_{t0})/\,t$$

where, W is the average total weight (g) at t days of trial. The net accumulation rate for each contaminant at each sampling day (NAR; ng g^{-1} of dw day^{-1}) was determined assuming that bivalves were exposed to steady conditions (i.e. continuous contaminant exposure, as well as seawater abiotic parameters) and using the following equation (Santana et al., 2017):

$$NAR_t = \frac{([cont_t] - [cont_{t0}])}{t}$$

where, [cont] is the average contaminant concentration in bivalve tissues (i.e. contaminant bioaccumulated) after t days of exposure.

The percentage of contaminant lost during the elimination phase of each trial, i.e. the elimination factor (EF;%) was calculated according to the following equation:

$$EF = 100 - \left(\frac{[cont_t]}{[cont_{t20}]} \text{ x } 100 \right)$$

where, [cont] is the average contaminant concentration in bivalve tissue after t days of elimination and [$cont_{t20}$] is the average contaminant by the end of the exposure phase, i.e. day 20 (Jebali et al., 2014). EF was considered to be 0 whenever [$cont_{t22,30\ and\ 40}$] higher than [$cont_{t20}$].

To perform statistical analysis, data were first tested for normality of distribution (Kolmogorov–Smirnov's test) and homogeneity of variance (Bartlett's test), and Log-transformed whenever necessary to comply with both assumptions of the ANOVA test. Then, two-way ANOVA test was used to check for the presence or absence of significant differences between contaminated treatments, with temperature (19 ºC or 23 ºC) and pH (8.0 units or 7.6 units) as variables. Post-hoc Tukey HSD test was subsequently carried out to identify such differences. Finally, potential correlations between CI, GR, NAR and EF were investigated by means of Pearson's correlation analysis. Statistical analyses were performed at a significance level of 0.05, using STATISTICA™ software (Version 7.0, StatSoft Inc., USA).

3. Results

3.1. Contaminant levels in seawater, feed and CTR bivalve samples (Trials I and II)

In Trial I, Decs 602, 603 and 604 were not detected in seawater samples (both CTR and CONT treatments with concentrations < LOD), neither in Japanese carpet shell clam samples (*R. philippinarum*) collected in CTR treatment (**Table 6.1.**). As for the enriched feed, despite equivalent amounts of Decs 602, 603 and 604 were added in order to reach the same final concentration (~50 ng g^{-1}), a remarkable loss of Dec 602 and Dec 604 occurred during feed preparation (final concentrations of 10.0 ± 0.4 ng g^{-1} and 1.0 ± 0.1 ng g^{-1} for Dec 602 and Dec 604, respectively; **Table 6.1.**). Such differences in Decs' concentrations were subsequently accounted for when analysing data. As previously described, TBBPA was not detected in the contaminant enriched feed, therefore, the bioaccumulation of this compound was subsequently assessed in Trial II.

In Trial II, none of the selected contaminants were detected in seawater samples collected in CTR treatment (i.e. TBBPA, PFOA and PFOS concentrations < LOD), except iAs (74.6 ± 3.3 µg L^{-1}; **Table 6.1.**). Such results translated into detectable levels of iAs in CTR mussel samples (10.9 ± 4.3 ng g^{-1}; **Table 6.1.**), which were subsequently taken into account when analysing data from CONT treatments. As for seawater samples from all CONT treatments, PFOA and PFOS concentrations were stable and around the nominal value set for these compounds. Conversely, iAs final concentrations were also steady by slightly above the nominal concentration defined for this element (around 150 µg L^{-1}), whereas lower TBBPA final concentrations were found in

seawater samples collected from treatments exposed to acidification (i.e. CONT+Acid = 4.0 ± 1.3 µg L^{-1} ; CONT+Acid+Warm = 2.0 ± 1.1 µg L^{-1}; **Table 6.1.**).

3.2. Trial I – Exposure to Dec 602, Dec 603 and Dec 604 via enriched feed

Figure 6.2. presents the concentrations of Decs 602, 603 and 604 in Japanese carpet shell clams' meat during 40 days of Trial I. During contaminant exposure, detectable levels of these three compounds were found in all clam samples collected from CONT treatments, with Dec 603 showing, overall, higher concentrations than Dec 602 and Dec 604, regardless of seawater temperature and pH (**Figure 6.2.**). Overall, warmer temperatures significantly increased Dec's 602 concentration, straight from the second day of exposure ($p < 0.01$; **Figure 6.2.** and **Table 6.2.**). Furthermore, exhibiting a less pronounced increase in the first days of exposure compared the other treatments, after 20 days of exposure, treatment CONT+Acid+Warm registered the maximum Dec 602 concentration (i.e. 0.19 ng g^{-1} dw, equivalent to a NAR = 0.010 ng g^{-1} day^{-1}; $p < 0.01$; **Figure 6.2.** and **Table 6.2.**). Subsequently, a reduction was observed immediately after 2 days of depuration (i.e. day 22) in all treatments. Yet, acidification acting alone (i.e. CONT+Acid) resulted in the lowest EF for this compound after 20 days of elimination period (78%; $p < 0.05$; **Table 6.2.**). As for Dec 603, lower pH levels, alone or combined with warmer temperatures, promoted significantly higher concentrations of this compound in clams' meat during the exposure phase (i.e. CONT+Acid = 1.53 ± 0.02 ng g^{-1} dw and CONT+Acid+Warm = 1.16 ± 0.01 ng g^{-1} dw), as well as during depuration (i.e. CONT+Acid = 0.23 ± 0.01 ng g^{-1} dw and CONT+Acid+Warm = 0.06 ng g^{-1} dw*;* $p < 0.01$; **Figure 6.2.**). Such trend resulted in significantly higher NARs and lower EFs in treatments CONT+Acid and CONT+Acid+Warm ($p < 0.05$; **Table 6.2.**). Warming alone (i.e. CONT+Warm) has also lead to significant higher levels of Dec 603 at the end of the exposure and until day 30 compared to treatment CONT (**Figure 6.2.** and **Table 6.2.**). Finally, during the 20 days of exposure, Dec 604 revealed significantly higher levels in bivalves subjected to warming and acidification acting alone (i.e. ~0.19 ng g^{-1} dw in CONT+Acid and CONT+Warm*;* $p < 0.01$), but not when both effects were combined (i.e. CONT+Acid+Warm; **Figure 6.2.** and **Table 6.2.**). Despite the statistically higher concentrations of Dec 604 in the treatment CONT+Warm at days 22 and 30, by the end of the clearance period the percentages of elimination of this compound were similar among treatments (**Table 6.2.**). Regarding animal condition (CI) and growth rate (GR; **Table 6.3.**), although the CIs of contaminated animals were not significantly different from non-contaminated ones (i.e. CTR) nor within CONT treatments, overall, bivalves exposed to warmer temperatures revealed significantly higher GR than those exposed to the reference temperature or to low pH alone, namely at days 10 (CONT+Warm; $p < 0.05$), 20 (CONT+Warm and CONT+Acid+Warm; $p < 0.05$) and 30

(CONT+Acid+Warm; $p < 0.05$) (**Table 6.3.**). Moreover, ignificant positive correlations were found between NAR of Decs 602, 603 and 604, and clams GR ($p < 0.05$; **Table 6.4.**).

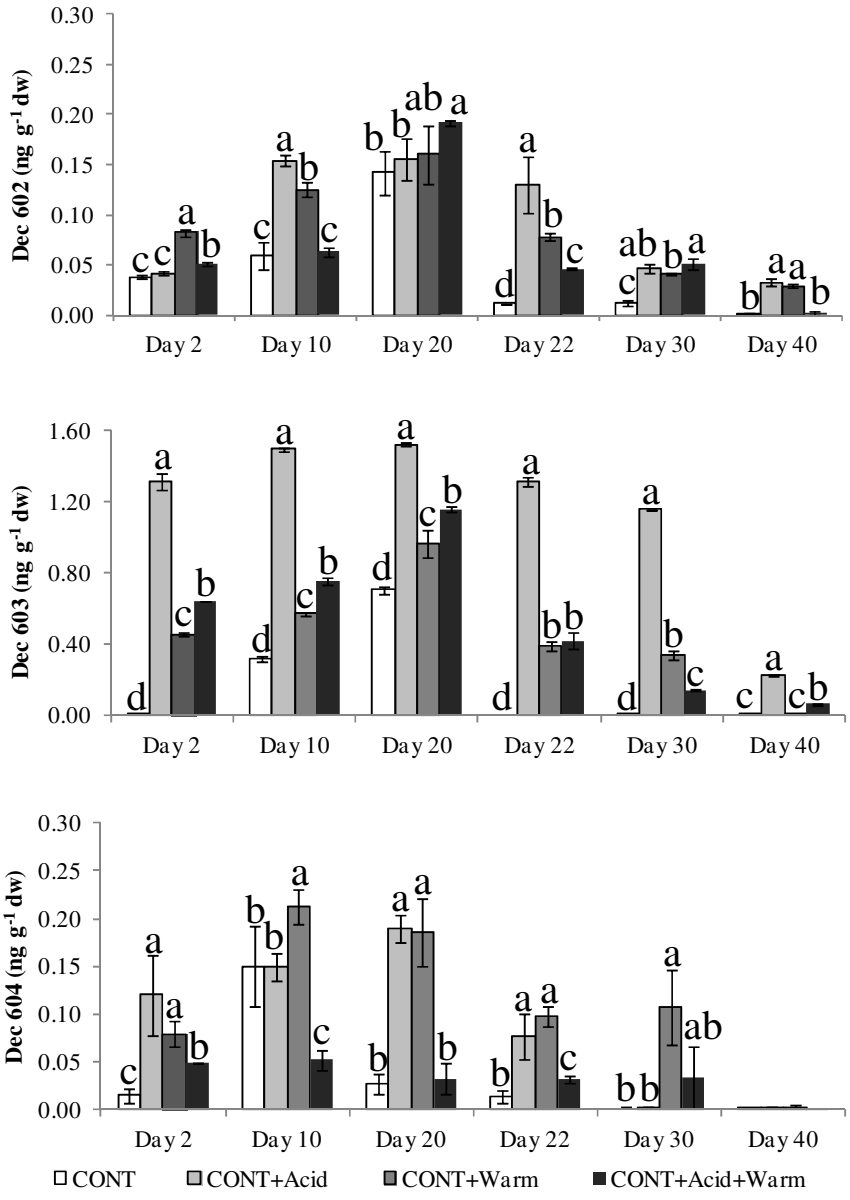

Figure 6.2. Dechloranes' concentrations (dw) in Japanese carpet shell clam samples (*R. philippinarum*) from each contaminated treatment during the 20 days exposure and 20 days of elimination in Trial I (mean ± standard deviation; $n = 3$). Different letters indicate significant differences between treatments for each day ($p < 0.05$). Abbreviations: CONT - contaminated, 19 ºC, 8.0 pH units; CONT+Acid - contaminated, 19 ºC, 7.6 pH units; CONT+Warm - contaminated, 23 ºC, 8.0 pH units; CONT+Acid+Warm - contaminated, 23 ºC, 7.6 pH units.

Table 6.2. Net accumulation rates (NAR; mean ± standard deviation) after 20 days of contaminant exposure and elimination factors (EF; mean ± standard deviation) after 20 days of elimination in contaminated treatments. In each column (and sampling day), different letters indicate significant differences between treatments ($p > 0.05$). Abbreviations: CONT - contaminated, 19 ºC, 8.0 pH units; CONT+Acid - contaminated, 19 ºC, 7.6 pH units; CONT+Warm -contaminated, 23 ºC, 8.0 pH units; CONT+Acid+Warm - contaminated, 23 ºC, 7.6 pH units.

		Dec 602		Dec 603		Dec 604		TBBPA		iAs		PFOA		PFOS	
Sampling day	**Treatment**	NAR ng g^{-1} dw day^{-1}	EF (%)	NAR ng g^{-1} dw day^{-1}	EF (%)	NAR ng g^{-1} dw day^{-1}	EF (%)	NAR ng g^{-1} dw day^{-1}	EF (%)	NAR ng g^{-1} dw day^{-1}	EF (%)	NAR ng g^{-1} dw day^{-1}	EF (%)	NAR ng g^{-1} dw day^{-1}	EF (%)
Day 20	CONT	0.007 ± 0.001^{b}	-	0.035 ± 0.001^{d}	-	0.001 ± 0.001^{b}	-	243.4 ± 15.8^{b}	-	35.1 ± 0.2^{a}	-	0.98 ± 0.11^{a}	-	113.1 ± 2.7^{a}	-
	CONT+Acid	0.007 ± 0.001^{b}	-	0.076 ± 0.001^{a}	-	0.010 ± 0.001^{a}	-	150.9 ± 2.6^{c}	-	18.1 ± 0.6^{b}	-	0.45 ± 0.01^{b}	-	95.5 ± 7.8^{b}	-
	CONT+Warm	0.008 ± 0.001^{ab}	-	0.048 ± 0.004^{c}	-	0.009 ± 0.002^{a}	-	256.5 ± 40.4^{ab}	-	17.6 ± 0.4^{b}	-	0.27 ± 0.07^{c}	-	73.8 ± 5.4^{c}	-
	CONT+Acid+Warm	0.010 ± 0.000^{a}	-	0.058 ± 0.001^{b}	-	0.002 ± 0.001^{b}	-	285.8 ± 9.0^{a}	-	7.3 ± 0.4^{c}	-	0.56 ± 0.12^{b}	-	112.6 ± 3.6^{a}	-
Day 40	CONT	-	99.3 ± 0.9^{a}	-	99.9 ± 0.1^{a}	-	100.0 ± 0.0	-	97.3 ± 0.3^{b}	-	42.2 ± 5.8^{c}	-	68.4 ± 3.5^{b}	-	97.8 ± 0.5^{ab}
	CONT+Acid	-	78.7 ± 5.2^{b}	-	85.2 ± 0.3^{c}	-	98.6 ± 2.0	-	96.1 ± 0.4^{b}	-	46.9 ± 1.5^{c}	-	88.3 ± 1.4^{a}	-	98.8 ± 0.1^{a}
	CONT+Warm	-	81.5 ± 4.2^{ab}	-	99.9 ± 0.2^{a}	-	99.0 ± 1.3	-	99.7 ± 0.1^{a}	-	71.3 ± 0.9^{a}	-	73.7 ± 1.3^{b}	-	97.0 ± 0.5^{b}
	CONT+Acid+Warm	-	99.2 ± 1.1^{a}	-	94.6 ± 0.2^{b}	-	99.7 ± 0.5	-	99.5 ± 0.1^{a}	-	52.4 ± 0.4^{b}	-	90.5 ± 1.5^{a}	-	97.4 ± 0.4^{b}

Table 6.3. Bivalves' condition index (CI) and growth rate (GR) during 40 days of trial (20 days exposure + 20 days elimination; mean ± standard deviation). In each column (and day), different letters indicate significant differences between treatments ($p > 0.05$). Abbreviations: CTR – control (non-contaminated), 19 ºC, 8.0 pH units; CONT - contaminated, 19 ºC, 8.0 pH units; CONT+Acid - contaminated, 19 ºC, 7.6 pH units; CONT+Warm -contaminated, 23 ºC, 8.0 pH units; CONT+Acid+Warm - contaminated, 23 ºC, 7.6 pH units.

		Trial I (*R. philippinarum*)		**Trial II (*M. galloprovincialis*)**	
		CI	GR (g day^{-1})	CI	GR (g day^{-1})
Day 0	All	6.92 ± 0.46	-	4.96 ± 0.66	-
Day 2	CTR	7.08 ± 0.48	1.06 ± 0.13	5.18 ± 0.28	0.59 ± 0.59^{ab}
	CONT	6.98 ± 0.48	0.90 ± 0.16	5.35 ± 0.16	0.25 ± 0.05^{b}
	CONT+Acid	7.30 ± 0.36	0.85 ± 0.11	5.13 ± 0.46	0.56 ± 0.18^{a}
	CONT+Warm	6.74 ± 0.36	1.34 ± 0.45	5.02 ± 0.54	0.63 ± 0.17^{a}
	CONT+Acid+Warm	7.52 ± 0.40	1.09 ± 0.25	5.11 ± 0.22	1.18 ± 0.77^{a}
Day 10	CTR	7.01 ± 0.33	0.18 ± 0.03^{b}	5.00 ± 0.41	0.50 ± 0.44
	CONT	6.84 ± 0.36	0.14 ± 0.01^{c}	5.23 ± 0.08	0.61 ± 0.36
	CONT+Acid	6.93 ± 0.53	0.13 ± 0.01^{c}	5.00 ± 0.67	0.83 ± 0.19
	CONT+Warm	7.54 ± 0.57	0.37 ± 0.03^{a}	5.21 ± 0.43	0.45 ± 0.33
	CONT+Acid+Warm	7.00 ± 1.16	0.23 ± 0.04^{b}	5.04 ± 1.67	0.71 ± 0.62
Day 20	CTR	7.11 ± 0.55	0.08 ± 0.01^{c}	5.25 ± 0.32	0.75 ± 0.18^{a}
	CONT	7.03 ± 0.32	0.09 ± 0.01^{bc}	5.14 ± 0.15	0.28 ± 0.11^{b}
	CONT+Acid	7.09 ± 0.51	0.06 ± 0.00^{d}	5.25 ± 0.23	0.11 ± 0.10^{b}
	CONT+Warm	6.96 ± 0.64	0.12 ± 0.01^{a}	5.25 ± 0.21	0.83 ± 0.12^{a}
	CONT+Acid+Warm	7.26 ± 0.27	0.11 ± 0.01^{a}	5.19 ± 0.53	0.79 ± 0.22^{a}
Day 22	CTR	7.23 ± 0.20	0.05 ± 0.01^{ab}	5.72 ± 0.72	1.43 ± 0.75^{a}
	CONT	7.32 ± 0.35	0.04 ± 0.01^{b}	5.62 ± 0.37	0.13 ± 0.12^{b}
	CONT+Acid	7.25 ± 1.04	0.08 ± 0.02^{a}	5.30 ± 0.24	1.19 ± 0.28^{b}
	CONT+Warm	7.26 ± 0.50	0.04 ± 0.01^{b}	5.63 ± 0.55	0.67 ± 0.54^{ab}
	CONT+Acid+Warm	7.46 ± 0.70	0.06 ± 0.01^{ab}	5.97 ± 0.90	0.82 ± 0.06^{ab}
Day 30	CTR	7.06 ± 0.43	0.03 ± 0.01^{ab}	5.73 ± 0.86^{ab}	1.01 ± 0.14^{a}
	CONT	7.13 ± 0.49	0.02 ± 0.01^{b}	5.68 ± 0.34^{ab}	0.19 ± 0.16^{b}
	CONT+Acid	7.28 ± 0.56	0.04 ± 0.00^{ab}	5.34 ± 0.14^{b}	1.22 ± 0.34^{a}
	CONT+Warm	7.12 ± 0.30	0.04 ± 0.02^{ab}	5.84 ± 0.17^{a}	0.33 ± 0.01^{b}
	CONT+Acid+Warm	7.11 ± 0.51	0.05 ± 0.01^{a}	5.37 ± 0.11^{b}	0.39 ± 0.22 ^{b}
Day 40	CTR	7.03 ± 0.53	0.01 ± 0.00	5.53 ± 0.26	0.72 ± 0.07^{a}
	CONT	7.10 ± 0.46	0.01 ± 0.01	5.40 ± 0.18	0.46 ± 0.01^{b}
	CONT+Acid	7.53 ± 0.35	0.02 ± 0.01	5.41 ± 0.28	0.19 ± 0.03^{c}
	CONT+Warm	7.57 ± 0.71	0.02 ± 0.01	5.62 ± 0.69	0.02 ± 0.00^{d}
	CONT+Acid+Warm	7.56 ± 1.31	0.02 ± 0.01	5.48 ± 0.24	0.01 ± 0.00^{d}

Table 6.4. Pearson's correlation coefficients between animal growth rate (GR), condition index (CI) and contaminants' net accumulation rates (NAR) and elimination factors (EF). Asterisks indicate significant correlations between variables ($p > 0.05$).

	r	
	NAR	EF
Trial I		
GR x CI	-0.64	
GR x Dec 602	0.90*	-0.21
CI x Dec 602	-0.24	-0.04
GR x Dec 603	0.66*	0.27
CI x Dec 603	0.12	-0.23
GR x Dec 604	0.77*	-0.36
CI x Dec 604	-0.02	0.03
Trial II		
GR x CI	-0.25	
GR x TBBPA	0.17	-0.14
CI x TBBPA	-0.49*	-0.02
GR x iAs	0.07	-0.46
CI x iAs	-0.35	0.11
GR x PFOA	0.04	-0.27
CI x PFOA	-0.36	0.10
GR x PFOS	0.25	0.19
CI x PFOS	-0.43*	-0.13

3.3. Trial II – Exposure to TBBPA, iAs, PFOA and PFOS via contaminated seawater

As shown in **Figure 6.3.**, all mussel samples collected from CONT treatments revealed detectable levels of TBBPA, iAs, PFOA and PFOS. Concerning TBBPA, despite the significantly higher concentrations found in treatment CONT at day 10 of the trial, by the end of the exposure phase (day 20) mussels exposed to warming combined with acidification showed the highest concentrations (i.e. treatment CONT+Acid+Warm = 5716 ± 179 ng g^{-1} dw), as well as the highest NARs for this compound ($p < 0.05$; **Figure 6.3.** and **Table 6.2.**). Furthermore, increased seawater temperature (with or without pH decrease) also led to a significantly higher elimination of TBBPA (~99% of TBBPA concentration at day 40 in these treatments; $p < 0.05$; **Table 6.2.**). Conversely, significantly lower concentrations of TBBPA were found in mussels subjected to lower pH alone. As far as iAs and PFOA are concerned, warm and acid (alone or combined) treatments revealed significantly lower levels compared to those found in specimens exposed to reference temperature and pH conditions during exposure (CONT = 711 ± 3 ng g^{-1} dw and 20 ± 2 ng g^{-1} dw, for iAs and PFOA respectively, at day 20; $p < 0.05$; **Figure 6.3.**).

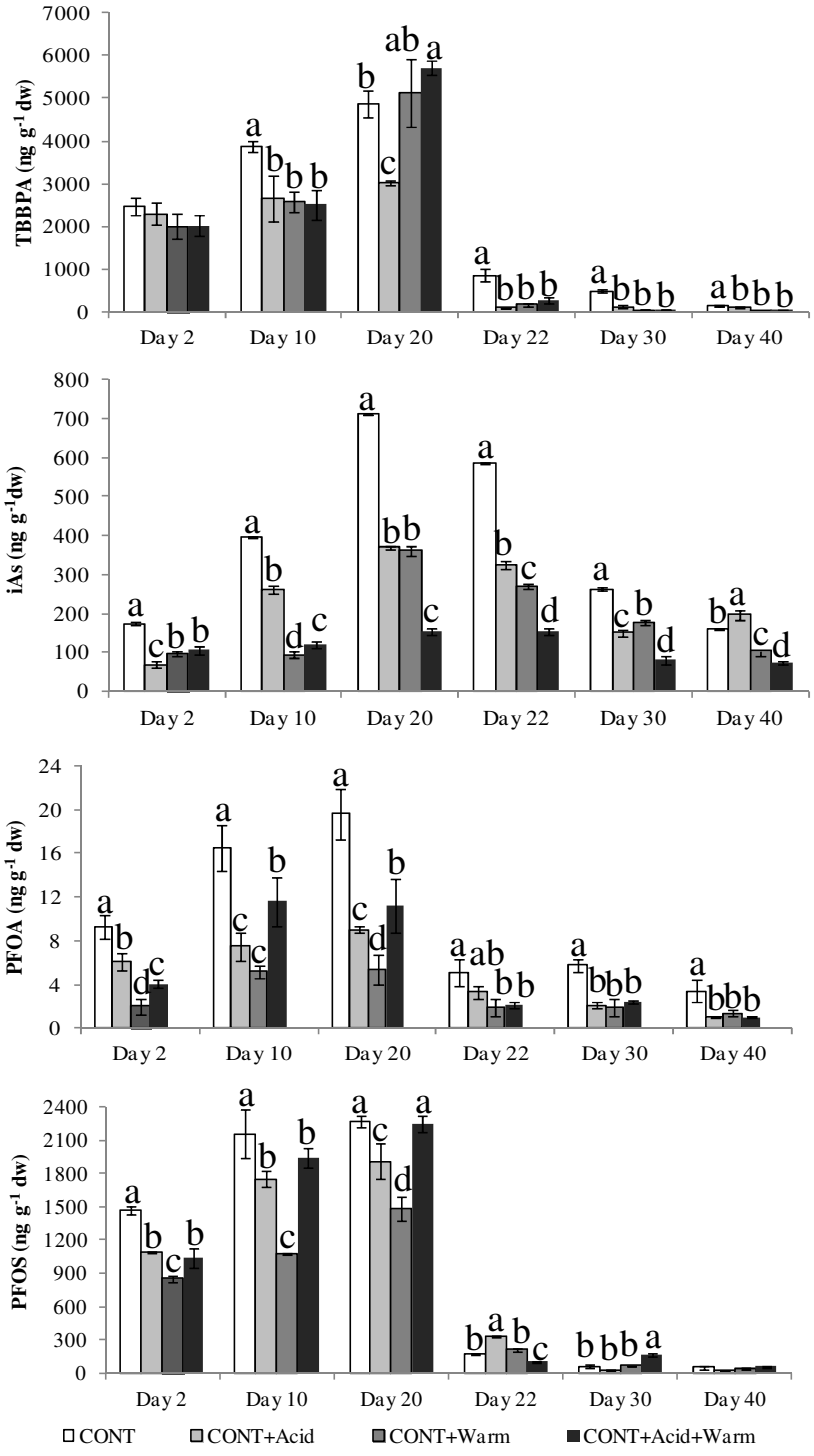

Figure 6.3. Contaminants' concentrations (dw) in Mediterranean mussel samples (*M. galloprovincialis*) from each contaminated treatment during the 20 days exposure and 20 days of elimination in Trial II (mean ± standard deviation; $n = 3$). Different letters indicate significant differences between treatments for each day ($p < 0.05$). Abbreviations: CONT - contaminated, 19 ºC, 8.0 pH units; CONT+Acid - contaminated, 19 ºC, 7.6 pH units; CONT+Warm - contaminated, 23 ºC, 8.0 pH units; CONT+Acid+Warm - contaminated, 23 ºC, 7.6 pH units.

As for the depuration period, iAs showed the highest EF in warming treatments (over 52% with or 71% without acidification; $p < 0.05$; **Table 6.2.**), whereas acidification promoted a higher elimination of PFOA (90% with or 88% without warming; $p < 0.05$; **Table 6.2.**). Finally, significantly lower PFOS concentrations were found in bivalves exposed to warming and acidification alone (i.e. CONT+Warm = 1476 ± 108 ng g^{-1} dw and CONT+Acid = 1910 ± 156 ng g^{-1} dw; $p < 0.01$; **Figure 6.3.**) compared to the CONT treatment (i.e. contaminated under reference temperature and pH), but not with the combination of both factors, whereas the highest percentages of elimination were found in CONT+Acid (99%; $p < 0.05$; **Table 6.2.**), though not statistically different than mussels from the CONT treatment. Animal CI also did not vary significantly in Trial II, with the exception of day 30, in which bivalves exposed to warming (CONT+Warm) showed significantly higher CI than those exposed to acidification (CONT+Acid and CONT+Acid+Warm; $p < 0.05$; **Table 6.3.**). Moreover, a significant negative correlation was found between CI and NAR of TBBPA and PFOS, but not for the remaining contaminants ($p < 0.05$; **Table 6.4.**). In general, non-contaminated animals revealed significantly higher GR than contaminated ones exposed to the same temperature and pH values (i.e. CTR *versus* CONT; $p < 0.05$; **Table 6.3.**). Yet, when exposed to warming alone or the combination of warming and lower pH, significantly higher mussels GR were observed compared to animals exposed to control temperature and pH (i.e. CONT), and to contaminated mussels under low pH during exposure (CONT+Acid). In contrast, by the end of the elimination period, treatments under reference temperature and pH (CTR and CONT) revealed statistically higher GR compared to the remaining treatments ($p < 0.05$; **Table 6.3.**).

4. Discussion

Starting with contaminant levels in the enriched feed (Trial I), results evidenced that compound stabilization difficulties occurred during feed preparation for Dec 602 and Dec 604, as well as for TBBPA, which was also intended to be studied using the first experimental approach (exposure via enriched feed) in the first place, as previously mentioned. Such feed stabilization difficulties can be possibly due to contaminant adsorption to the equipments used during feed preparation and/or compound degradation (e.g. mechanic degradation or due to heat exposure). Furthermore, the considerable loss of TBBPA compared to Decs may be related to the fact that this compound is known to be less persistent and easily degraded, being subsequently transformed in BPA due to the loss of its bromine groups. As for contaminant levels in seawater samples, in Trial I results evidenced that: i) contaminant leaching from feed to seawater did not occur in Trial I; and ii) no other source of external chemical contamination apart from the enriched feed occurred.

Concerning seawater samples (Trial II), the levels of iAs found in CTR treatment (which translated in detectable concentrations of this compound in CTR mussels, as well as in iAs concentrations in CONT treatments above the defined nominal value) are most likely due to the presence of this compound in the maintenance feed used in these trials. On the other hand, the lower TBBPA concentrations observed in seawater samples from acid treatments (CONT+Acid and CONT+Acid+Warm) may be related to the fact that, as most ionisable compounds, TBBPA can alternate from molecular to ionic forms and vice-versa, precipitate or even be degraded according to the surrounding pH level.

Regarding bivalve samples from both trials, the higher concentrations of Dec 603 compared to Decs 602 and 604 found in clams' edible tissues (all CONT treatments) during the exposure phase can be partially attributed to the lower daily exposures of Dec 602 (~5 times) and even lower of Dec 604 (~50 times) compared to Dec 603, due to the considerable loss of these compounds during feed preparation (**Table 6.1.**), as previously mentioned. It should be noted that differences in Decs' bioaccumulation can also be related to distinct bioavailability (i.e. readiness to be absorbed at an organism's digestive epithelia) of each compound, as it has been also reported in wild biota, with Dec 602 being often found in marine species at higher levels compared to other Dec's (i.e. Dec 603, Dec 604 and Dec Plus; Feo et al., 2012; Aznar-Alemany et al., 2017). The same fact can also justify the discrepancy in concentration ranges of PFOA a PFOS observed in Trial II, despite the similar concentrations in seawater, with the second exhibiting much higher NAR during the exposure phase, regardless of the treatment, than the first one. In fact, the bioaccumulation of PFCs in biota, as well as their adsorption to sediments has been previously shown to be dependent on the length of the perfluorinated tail (e.g. Martin et al., 2004). A recent field study performed in marine biota from different taxonomic groups concluded that the bioaccumulation and elimination of these compounds is largely dependent on species and compound, with fish species showing, in general, higher levels of PFOS than PFOA, whereas bivalves showed the opposite trend, thus, contrasting the present findings (Hong et al., 2015).

As for the effects of temperature and pH, the current lack of empirical studies focusing on the bioaccumulation and toxicity of emerging contaminants, hinders adequate comparisons of the findings acquired in this first study with previous literature on contaminants' kinetics. Yet, the different trends observed in bivalves exposed to increased seawater temperature and reduced pH in this study highlighted the urgent need to consider the interactions between multiple stressors when assessing the potential environmental and human health risks of emerging contaminants' exposure, especially in the context of climate change.

By enhancing biota's metabolism, thus, increasing species ventilation and feeding rates in response to higher metabolic demands (Dijkstra et al., 2013), warmer temperatures can likely translate into higher contaminant bioaccumulation, as well as increased contaminant

metabolization and excretion (e.g. Maulvault et al. 2016; Sampaio et al., 2016). Hence, such metabolic enhancement may justify the increased bioaccumulation of some compounds under warmer seawater temperatures, particularly those with long half-lives, such as Dec 602, Dec 603 and Dec 604, which are more likely to build up their concentrations in tissues under continuous exposure conditions due to their lipophilic behaviour and low elimination rates (Shen et al., 2010). Similarly, a recent study performed with juvenile seabass (*Dicentrachus labrax*) exposed to methylmurcury (MeHg) from dietary sources, also revealed higher MeHg bioaccumulation at warmer temperatures, as well as diminished ability to detoxify this persistent and biomagnifying pollutant (Maulvault et al., 2016). On the other hand, enhanced metabolic rates due to warming may translate into the opposite trend for compounds with lower ability to persist in biological compartments, i.e. those with shorter half-lives, which can be transformed and subsequently excreted at faster rates (i.e. within 24 to 72 hours; e.g. Vanden Heuvel et al., 1991; Knudsen et al., 2007; WHO, 2001), as observed in the present study for some compounds, such as iAs, TBBPA and PFOA. Furthermore, animal growth should also be accounted when interpreting contaminants' bioaccumulation in warmer environments, as increased feeding rates to support enhanced metabolic demands can also result in greater growth, leading to reduced contaminant bioaccumulation through somatic growth dilution (Dijkstra et al., 2013). Yet, despite few variations in GR, particularly in CONT+Warm and CONT+Warm+Acid treatments, the opposite trend was observed in terms of Decs' bioaccumulation (i.e. GR positively correlated with NARs), whereas there was no clear relation between GR and the bioaccumulation of the other compounds.

Concerning the effect of acidification, as observed in Decs 603 and 604, increased contaminant bioaccumulation has also been reported in some studies due to metabolic changes under hypercapnia (e.g. Rosa et al., 2016; Sampaio et al., 2016, 2018), as well as, damages in tissues' apical epithelial membrane that facilitate contaminant penetration into cells (Freitas et al., 2016; Sampaio et al., 2016, 2018; Shi et al., 2016; Velez et al., 2016). On the other hand, lower contaminant elimination at reduced pH levels has also been previously described due to the fact that bivalves possess a valve closing strategy of defence when exposed to stressors (i.e. surrounding pH outside species' optimal range; and/or contaminants exposure), thus, preventing the uptake of contaminants, as well as the excretion of compounds and their metabolites into the environment (Freitas et al., 2016; Velez et al., 2016). Such argument may justify the lower elimination of Dec 602, Dec 603, TBBPA and iAs under acid conditions. Yet, such physiological responses under reduced pH conditions seem to be reversed when warming is also added to the equation. Also worth mentioning, particularly in what concerns metals and other ionizable compounds, is the fact that the surrounding seawater pH levels strongly influences the chemistry, speciation and, thus, the availability of these compounds (e.g. Shi et al., 2016; Velez et al., 2016). Contrasting the results observed in the present study, Velez et al. (2016) reported an increase in iAs bioaccumulation at lower pH, justifying their results with the fact that the uptake of this

element occurs via the phosphate transport systems (such as Na^+ and K^+-ATPase) which are also involved in pH osmoregulation of estuarine biota, thus probably leading to the competition for the same transport mechanisms (Monserrat et al., 2007). Yet, possible justifications for such differences in iAs bioaccumulation patterns include: i) different iAs forms used in each study (H_3AsO_4 in this study; $Na_2HAs_4^-$ in Velez et al., 2016); ii) iAs bioaccumulation depends on the tested pH level (8.0 and 7.6 pH units in the present study and 7.8 and 7.3 pH units in Velez et al., 2016); iii) iAs bioaccumulation may be a species dependent mechanism (*M. galloprovincialis* in the present study and *R. philippinarum* in Velez et al., 2016), given distinct filtration rates of bivalve species, as well as the ability for some species to keep valves closed as a strategy of protection against the exposure to contaminants and/or low pH (Freitas et al., 2016; Velez et al., 2016). As for the bioaccumulation and elimination of PFOA and PFOS, which was also affected by pH level, studies involving biota are extremely scarce and, to the authors' best knowledge, the present report is the first to explore the effect of pH on the bioaccumulation of these contaminants. Hence, despite it is not possible to compare the present data with previous reports on marine biota, both of these PFCs have been pointed out intensively in the literature to alter from molecular forms into ionic dissolved forms according to the surrounding pH conditions, with higher pH facilitating the uptake of adsorbed molecular forms (e.g. Higgins and Luthy, 2006; Wang et al., 2012). Furthermore, unlike other pollutants, PFOA and PFOS do not primarily accumulate in adipose tissues, but rather bind to proteins, such as albumin, which are mainly present in blood, liver and eggs (e.g. Martin et al., 2003). Since albumin is involved in organisms' osmoregulation, being responsible for controlling the osmotic pressure in tissues, changes in surrounding pH may interfere with albumin ability to bind to PFOS and PFOA, thus, resulting in lower tissue bioaccumulation. This pattern has been described in several pharmacokinetic studies involving vertebrate species and different chemical compounds (Kim et al., 1999; Hinderling and Hartmann, 2005). Regarding the combination of warmer temperature and lower pH, the distinct patterns compared to those observed when both stressors acted in isolation (e.g. after 20 days of exposure, Dec 604 and iAs: CONT+Acid and CONT+Warm significantly higher than CONT+Warm+Acid; PFOA and PFOS: CONT+Acid and CONT+Warm significantly lower than CONT+Warm+Acid), emphasized the importance of considering the interactions between different abiotic stressors in studies focused on contaminants' bioaccumulation kinetics. Particularly concerning TBBPA, the bioaccumulation trends observed for this compound (i.e. increased in CONT+Acid+Warm during the last 10 days of exposure) suggest that, though bivalves inhabiting estuarine, intertidal, and subtidal areas, like R. *philippinarum* and *M. galloprovincialis*, have developed strategies to cope with the presence of multiple environmental stressors at the same time (i.e. pollution and wide ranges of abiotic conditions; Lannig et al., 2010), such physiological plasticity may: i) be time-limited, with species resilience to stress being

committed under long-term and continuous exposure conditions (Belivermis et al., 2016); ii) have pushed bivalves' resilience to the edge when both stressors were combined, given the high energetic costs required to simultaneously adapt to warmer seawater and lower pH (Lannig et al., 2010), thus compromising bivalve's ability to cope with TBBPA exposure.

To sum up, in a more generalized way, despite the different bioaccumulation and elimination patterns observed according to each compound, overall: i) warming (acting alone) seemed to play a key role for contaminants with longer half-lives, such Decs and TBBPA, enhancing their bioaccumulation; ii) acidification, on the other hand, seemed to particularly influence the bioaccumulation/elimination of ionisable compounds, such as TBBPA, iAs, PFOA and PFOS, impairing their bioaccumulation; iii) when both abiotic stressors are combined, these previously described effects are accentuated in the case of some contaminants(e.g. even higher Dec 602 bioaccumulation and even lower iAs bioaccumulation when both stressors are combined), but reversed for others (e.g. lower TBBPA and PFOS bioaccumulation in treatments simulating warming and acidification in isolation).

A direct link can also be established between the increased bioaccumulation and/or impaired detoxification of selected emerging contaminants, particularly Dec 602, 603, 604 and TBBPA under warmer temperatures and/or lower pH levels and the potential implications of these findings in seafood safety. Despite the presence of these compounds in seafood still remains unregulated, nor recommendations concerning tolerable limits of intake have been established due to the current lack of toxicological studies (e.g. EFSA, 2012), an adequate hazard assessment and risk analysis cannot be performed in the present study. Yet, the results suggest that human exposure to Decs and TBBPA through the consumption of seafood may increase under higher temperatures and lower pH. Such findings can be of particular concern, considering the fact that marine bivalves are important food resources from the economical and nutritional point of view, being among the most frequently consumed seafood species worldwide (FAO, 2016).

5. Conclusions

As clearly evidenced in this first study, temperature and pH can strongly affect the bioaccumulation and elimination patterns of emerging chemical contaminants in marine organisms, by affecting seawater physical and chemical properties, as well as animal metabolism and physiological responses. Overall, while warming in isolation enhanced the bioaccumulation of contaminants, particularly those with longer half-lives (e.g. Decs and TBBPA), acidification seemed to have a preponderant role on the bioaccumulation/elimination of ionisable compounds, reducing the bioaccumulation of TBBPA, iAs, PFOA and PFOS. Noteworthy, the combination of warming and acidification seemed to have reversed the effects promoted by both stressors

acting alone, in the specific cases of TBBPA and PFOS (both ionisable), but not in the remaining compounds.

Despite variations were found according to the chemical compound, these results provide new insights on emerging contaminants' kinetics in bivalves maintained at higher temperatures and lower pH, and the potential human health risks associated to their consumption in the future. It also strengthens the need to carry out greater research efforts to understand how multiple environmental stressors interact with each other. Given the distinct behaviour of each contaminant and the different trends observed when warmer seawater and lower pH acted alone or in combination, future research should take into consideration regional trends (i.e. abiotic factors, pollution levels and diversity of contaminants) when addressing the expected effects of climate change on the kinetics of emerging contaminants, as the alterations of environmental conditions will certainly not affect marine ecosystems in the same way across the planet. Furthermore, because environmental stressors will unlikely occur in isolation, or all at once, different combinations of contaminant mixtures and abiotic conditions (exploring less pronounced to more severe scenarios) should be investigated to have a broader view of the toxicological impacts of climate change. Strengthening the knowledge on this matter will allow to incorporate the effects of climate change in future national and international regulations and set recommendations for human exposure to emerging contaminants, as well as to develop mitigation strategies to assure seafood safety in tomorrow's ocean.

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CHAPTER 7.

GENERAL DISCUSSION

General Discussion

Climate change and chemical contamination are two of the greatest environmental concerns that Man faces at the present. Both stressors are expected to occur either in isolation or simultaneously, depending on the region of the planet, with their combined effects representing additional challenges to the resilience of marine ecosystems, thus, potentially compromising the safety and sustainability of fisheries and aquaculture resources. In this way, this PhD thesis intended to provide a valuable contribution to the overall understanding of climate change impacts from the marine ecotoxicology and seafood safety perspectives, by assessing the combined effects of seawater warming and acidification on different emerging chemical contaminants (ECCs) with distinct chemical and toxicological attributes. In the end, the knowledge acquired throughout this PhD work can be seen as an "additional knowledge-based evidence" towards the need of effective regulation of ECCs in the environment (i.e. their discharges and/or efficient removal from wastewater) and of greenhouse gases emissions (that can speed up and/or intensify climate change effects). It also supports the need to develop mitigation strategies that will assure a sustainable management of marine resources in tomorrow's ocean.

In the objectives section (**Chapter 1.4.1.**), three research questions were initially proposed. **Chapter 7.** presents a summary of the main findings of this PhD thesis and a concrete answer to each of these questions. Starting with the first question:

1. *Will warming and/or acidification affect ECCs' bioaccumulation and elimination mechanisms in marine biota?*

Yes. Results gathered in this PhD thesis showed that warming and/or acidification indeed affect ECCs' bioaccumulation and elimination mechanisms in marine fish and bivalve species. Yet, different patterns were observed according to compound and/or abiotic stressor (**Table 7.1.**).

As mentioned throughout this PhD thesis, by enhancing biotas' metabolism, warmer temperatures can exacerbate ECCs' bioaccumulation and, at the same time, facilitate their metabolization and subsequent elimination (e.g. Noyes et al., 2009; Marques et al., 2010; Dijkstra et al., 2013; Serra-Compte et al., 2018). As such, overall, warming increased the bioaccumulation of ECCs with a more lipophilic behaviour and longer half-lives, such as MeHg and Decs (Shen et al., 2010; Sverko et al., 2011; Jo et al., 2015), while ECCs' tissue burdens tended to decrease for ionic compounds that are less hydrophobic and persistent (i.e. iAs, VFX, TCS and DCF, which was not detected in its parental form in none of the samples), being promptly metabolized/detoxified by marine biota (ECCs chemical properties and toxicity data are presented in **Chapter 1.** and **Table 1.1.**).

Table 7.1. Summary of climate change effects on ECCs' bioaccumulation and elimination mechanisms in marine biota, presented as the percentage (%) of change in relation to the ECC content observed under the reference temperature and pCO_2 conditions. ↑ or ↓ indicate significant increase or decrease, respectively, in relation to the contaminated treatment simulating the reference temperature and pCO_2 conditions. Moreover, values in red or green represent a negative change or positive change from the toxicological point of view, respectively. Abbreviations: MeHg, methylmercury; iAs, inorganic arsenic; DCF, diclofenac; VFX, venlafaxine; TCS, triclosan; TBBPA, tetrabromobisphenol A; Dec, dechlorane; PFOS, perfluorooctanesulfonic acid; PFOA, perfluorooctanoic acid; nd, not determined; ns, not significantly different (see statistical results in **Chapters 2.-6.**).

			Warming			Acidification			Warming+Acidification		
Biaccumulation	**Toxic elements**	**MeHg**	fish muscle: ↑22.3	fish liver: ns	fish brain: ↑50.6	nd	nd	nd	nd	nd	nd
		iAs	Bivalve: ↓49.3			Bivalve: ↓47.9			Bivalve: ↓78.3		
	PPCPs	**DCF**	nd	nd	nd	nd	nd	nd	nd	nd	nd
		VFX	fish muscle: ns	fish liver: ↓38.3	fish brain: ns	fish muscle: ns	fish liver: ↓45.4	fish brain: ns	fish muscle: ns	fish liver: ↓56.0	fish brain: ns
		TCS	fish muscle: ↓85.0	fish liver: ↓83.7	fish brain: ↓52.4	fish muscle: ↓63.0	fish liver: ↓66.0	fish brain: ns	fish muscle: ↓50.6	fish liver: ↓81.4	fish brain: ↓47.0
	FRs	**TBBPA**	Bivalve: ns			Bivalve: ↓38.0			Bivalve: ↑17.4		
		Dec 602	Bivalve: ns			Bivalve: ns			Bivalve: ↑35.2		
		Dec 603	Bivalve: ↑36.9			Bivalve: ↑>100			Bivalve: ↑64.5		
		Dec 604	Bivalve: ↑>100			Bivalve: ↑>100			Bivalve: ns		
	PFCs	**PFOS**	Bivalve: ↓34.7			Bivalve: ↓15.6			Bivalve: ns		
		PFOA	Bivalve: ↓72.7			Bivalve: ↓54.1			Bivalve: ↓42.6		
Elimination	**Toxic elements**	**MeHg**	fish muscle: ns	fish liver: ↓13.9	fish brain: ns	nd	nd	nd	nd	nd	nd
		iAs	Bivalve: ↑29.1			Bivalve: ns			Bivalve: ↑10.2		
	PPCPs	**DCF**	nd	nd	nd	nd	nd	nd	nd	nd	nd
		VFX	fish muscle: ns	fish liver: ↓23.9	fish brain: ns	fish muscle: ↓100.0	fish liver: ↓32.5	fish brain: ↓41.4	fish muscle: ns	fish liver: ↓38.1	fish brain: ns
		TCS	nd	nd	nd	nd	nd	nd	nd	nd	nd
	FRs	**TBBPA**	Bivalve: ↑2.4			Bivalve: ns			Bivalve: ↑2.2		
		Dec 602	Bivalve: ns			Bivalve: ↓20.7			Bivalve: ns		
		Dec 603	Bivalve: ns			Bivalve: ↓14.7			Bivalve: ↓5.3		
		Dec 604	Bivalve: ns			Bivalve: ns			Bivalve: ns		
	PFCs	**PFOS**	Bivalve: ns			Bivalve: ns			Bivalve: ns		
		PFOA	Bivalve: ns			Bivalve: ↑20.0			Bivalve: ↓22.1		

In the specific case of VFX, compound bioaccumulation and elimination (in its parental form) diminished with warming and, especially, acidification. Such reduction could have been a result of somatic growth dilution (which is linked to the enhanced animal growth rates; e.g. Dijstra et al., 2013; Maulvault et al., 2016; Anacleto et al., 2018) combined with altered physiological mechanisms of xenobiotics' biotransformation and excretion (Serra-Compte et al., 2018), since most of the animals' energy was being channelled towards growth. Matching this argument, Serra-Compte et al. (2018) reported a decrease in VFX bioaccumulation and metabolization in mussels (*M. galloprovincialis*) exposed to acidification, which was mostly attributed to the physiological impairments elicited by this abiotic stressor.

PFOS and PFOA constituted a special case, as their low degradability (i.e. no biotransformation products of PFOS and PFOA were identified, so far, in *in vivo* models), extended half-life and relatively poor water solubility (EFSA, 2008) would suggest, beforehand, a higher bioaccumulation of these compounds in biota's tissues at warmer temperatures. Instead, a significant reduction of these ECCs was observed in bivalves exposed to warming, and this was most likely because, in the face of stressful surrounding conditions (e.g. pollution, abiotic variations), bivalves tend to keep their valves closed, thus, limiting/preventing not only the accumulation of xenobiotics, but also their metabolization and/or release to the environment (Freitas et al., 2016; Velez et al., 2016). This hypothesis may explain the decrease in PFOS and PFOA bioaccumulation under both warming and/or acidification, as well as the lower elimination of PFOA (**Table 7.1.**). In addition, it points out the fact that the bioaccumulation of PFCs (and of the remaining studied ECCs families) depends not only on species' ecological strategies (for instance, fish *versus* bivalves; Hong et al., 2015), but also on ECCs' exposure concentration and pathways, as both interfere with compound bioavailability (e.g. Memmert et al., 2013; Zenker et al., 2014; Silva et al., 2016; Maulvault et al., 2018b).

Acidification also seemed to have a preponderant role in the bioaccumulation of ionisable ECCs, leading to lower tissue burdens of VFX, TCS, TBBPA, iAs, PFOA and PFOS. This reduction can be attributed to different factors, such as: i) the energy required to maintain animal homeostasis under acidified conditions could have affected animal biological activities, translating into lower ECCs' uptake and/or metabolization rates; ii) ionisable ECCs can alternate between molecular forms according to the surrounding pH conditions, with tissue membranes being, in general, less permeable to ionized forms (Orvos et al., 2002; Rowett et al., 2016; Serra-Compte et al., 2018); and iii) in the specific case of bivalves, as mentioned above, the valve closing defence strategy might have prevented these species from bioaccumulating ECCs (Freitas et al., 2016; Velez et al., 2016). It should be also stressed that, contrasting with other studied ECCs, TBBPA bioaccumulation in bivalve species was enhanced by the combination of warming and acidification (but not by acidification alone). Since in this trial bivalves were exposed to TBBPA via water, such exacerbation of compound tissue burdens could have been related to an

alternation of TBBPA's molecular forms potentiated by the combination of increased temperature and lower pH levels in seawater (TBBPA's acid dissociation constant, pKa, has been estimated to be ~7.5; EFSA, 2011). On the other hand, the combination of thermal stress and hypercapnia may have also resulted in substantial tissue damage in bivalves, given the severity of such stress conditions, therefore, facilitating TBBPA's penetration into cells.

From a human perspective, the above mentioned changes elicited by seawater warming and acidification on marine organisms' ability to bioaccumulate and eliminate ECCs can be directly linked to seafood safety aspects, therefore, bringing up the second question posed in the beginning of this PhD thesis:

2. *Will seafood consumers be at a greater risk in tomorrow's ocean?*

The answer to this question is not straightforward, as the results gathered in this PhD thesis showed that the extent and direction of the changes elicited in ECCs' bioaccumulation and elimination mechanisms strongly **depend on the compound, marine species** (i.e. ecological attributes, e.g. feeding habits, position on the trophic chain and intraspecific mechanisms to cope with stressful environmental conditions), **and regional trends** (i.e. ECCs loads and profiles, as well as type/severity of climate change effects expected in a particular geographic area). In other words, and starting with the compound-dependency factor, the present results suggest that, in a climate change context, the risk of human dietary exposure to MeHg and FRs (Decs and TBBPA) through the consumption of contaminated seafood will increase, whereas the risk seems to be reduced in the case of iAs, PPCPs (TCS and VFX; no adequate information was obtained for DCF) and PFCs (PFOS and PFOA). However, such risk reduction can be fallacious because, as described above, it might be associated with an altered metabolization of parental ECCs under climate change-related stressors, therefore, meaning that an increased accumulation of more toxic and persistent metabolites may take place alongside for some contaminants. In accordance with this hypothesis, Serra-Compte et al. (2018) observed that higher pCO_2 levels impaired the biotransformation of VFX and its primary and most active metabolite O-desmethyl-VFX into less toxic compounds (i.e. N-desmethyl-VFX and NO-didesmethyl-VFX), therefore, suggesting that the toxicity of this antidepressant to marine bivalves is enhanced by ocean acidification. Despite the information regarding ECCs metabolization in marine fish and bivalves is extremely scarce, in mammals the metabolism of iAs involves the reduction of pentavalent As (AsV) into the trivalent As (AsIII) through the glutathione biotransformation pathway, with this second species being more toxic (i.e. more easily bound to proteins and subsequently transported across cell membranes) than the first (Thomas et al., 2001; Moulin et al., 2015). Similarly, despite TCS's half-life (parent compound) is estimated to be longer than other PPCPs, such as VFX or DCF, once uptaken, this compound also readily undergoes a series of metabolization steps involving

glucuronidation and sulfonation (Dhillon et al., 2015). Yet, no information is currently available in what concerns the toxicity of glucuronidated and sulfonated TCS conjugates to marine organisms. Although the trial focused on DCF bioaccumulation did not yield positive results and, thus, this matter cannot be appropriately discussed in this PhD thesis, it should be noted that this compound is also known to be rapidly metabolized (at the liver of vertebrate organisms), being subsequently transformed into different phase I and phase II metabolites, some of which (e.g. 4′-hydroxy-DCF) evidencing (in mammals) equal or even higher toxicity than the parental (unchanged) DCF (Syed et al., 2016).

As for the species-dependency factor, despite during this PhD research work it was impossible (due to time, budget and logistic constraints) to evaluate the bioaccumulation and elimination mechanisms of all tested ECCs in fish and bivalve model organisms, the data acquired with these preliminary trials suggests that climate change-related stressors will not affect these species in the same way, given their distinct ecological features. For instance, bivalves' valve closing reflex under stressful abiotic conditions might have contributed to the lower absorption of iAs, TBBPA (only under acidification) and PFCs from contaminated seawater. Yet, fish do not possess this strategy and, as such, different bioaccumulation patterns may take place when these species are exposed to the same ECCs, thus representing distinct risks to seafood consumers. On the other hand, from the ECCs' detoxification/elimination point of view, valve closing may also constitute a drawback to bivalve species that are neither able to release metabolized ECCs' forms to the environment nor to perform gas exchanges. By limiting gas exchanges, internal hypoxia may occur, forcing bivalves to experience impaired metabolic rates and physiological condition when they remain closed for extended periods of time. This may, in turn, emphasize ECCs' toxicity to these species (Carregosa et al., 2014; Correia et al., 2015) and, ultimately, lead to increased human exposure to these compounds (unchanged and/or biotransformed) when consuming contaminated bivalves. Moreover, climate change effects may also affect ECCs' bioaccumulation/elimination patterns in different ways among fish species and, thus constitute distinct risks to seafood consumers, depending on animals' feeding habits (e.g. predatory fish species with long life-cycles are more likely to accumulate high levels of persistent ECCs, such as MeHg, FRs and PFCs, in a warming context than small herbivorous species with shorter life cycles), distribution (e.g. species that spend longer periods of time in coastal areas subjected to stronger abiotic variations and inputs of ECCs *versus* species that mostly live in the open ocean) and habitat (pelagic species that are more active, have faster metabolic rates and feed in the water column *versus* benthic species that are less active, have lower metabolic rates and are in constant contact with contaminated sediments).

The distinct bioaccumulation and elimination patterns according to the ECC and climate change scenario (i.e. warming and acidification acting in isolation or simultaneously) also reveal that the extent to which the safety of seafood consumers will be affected in tomorrow's ocean

depends on various geographical factors. Such regional-dependency is due to the fact that neither chemical contamination, climate change effects, nor the adaptive and regulatory actions implemented to tackle both environmental stressors will be the same across the planet (IPCC, 2014; FAO, 2018). For instance, since the present results evidenced that warmer temperatures can facilitate the bioaccumulation of persistent organic pollutants (e.g. MeHg and FRs), special attention should be given to the environmental presence of these ECCs in geographic regions that are expected to be particularly fustigated by warming, namely coastal areas within the Northern Hemisphere, such as the Mediterranean, Black, North, Barents and Caspian seas (see **Chapter 1.** and **Figure 1.2.**). Conversely, as acidification may enhance the biotransformation of ionic ECCs into different metabolites (e.g. iAs, PPCPs and, potentially, PFCs), for which the toxicological information is still limited, careful monitoring of these ECCs should be implemented in higher latitude regions that are expected to experience a more pronounced decrease in the average seawater pH level compared to tropical areas (see **Chapter 1. Figure 1.2.**). Apart from monitoring the occurrence of these environmental stressors, accurately estimating the impacts of climate change in seafood safety will also require additional information specifically about the seafood trade chain, like the production (i.e. seafood species that are predominantly caught in fishing areas or farmed) and consumption patterns (i.e. consumers' preference and frequency of seafood consumption) within different countries, as these aspects can considerably vary from one region of the world to another, thus, being determinant factors to the risk assessment of human exposure to ECCs through seafood consumption (FAO, 2018).

In addition to the human perspective (i.e. seafood safety), the present research work also intended to contribute to the overall understanding of the ecological implications of climate change. Therefore, a third (and final) question was raised in the beginning of this PhD thesis:

3. *Will warming and/or acidification affect marine species' ecotoxicological responses to ECCs?*

Yes. Although it was not possible to investigate all endpoints in every trial preformed (the endpoints were selected according to the mode of action and expected toxicological effect of each ECC), preliminary results acquired in this PhD thesis showed that the way marine fish cope with the exposure to ECCs (e.g. MeHg, DCF, VFX and TCS) is strongly influenced by the surrounding temperature and pCO_2 conditions. Yet, the combination of these environmental stressors can elicit distinct effects (in terms of direction, i.e. up- or down-regulations, and extent/severity), at the different organizational levels (whole organism, organ or biochemical levels) and according to the ECC and type/severity of climate change effects (i.e. whether warming and acidification act in isolation or in combination). **Table 7.2.** presents a summary of the interactive effects that occur due to the co-exposure to ECCs and climate change-related stressors on a whole organism context.

Starting with the three selected indexes of animal fitness, while DCF and TCS co-exposed with warming and/or acidification significantly altered fish condition (K) and hepatosomatic (HSI) indexes, the same was not observed in fish exposed to MeHg or VFX. Furthermore, regardless of the ECC, no significant differences between contaminated fish exposed to the reference temperature and pCO_2 levels and those under different climate change scenarios were observed in terms of fish brain to body mass ratio (BB_{ratio}; **Table 7.2.**). In fact, as described in **Chapters 2.-5.**, the majority of changes with respect to these indexes were mostly found between non-contaminated and contaminated fish (i.e. regardless of temperature and pCO_2 conditions). This was most likely related to the fact that the simulated experimental conditions corresponded to worst-case scenarios of climate change effects (as well as of ECCs' contamination), but were still kept as realistic as possible (of course, taking into consideration the limitations associated with experimental laboratory conditions, which cannot exactly mimic what happens in nature). In other words, the experimental designs used in all trials attempted to simulate sub-lethal stress conditions, which were expected to trigger fish responses to a realistic and visible (during the timeline of each trial) extent, but not leading to total physiological collapse, drastically compromising animal survival (no mortality was observed in all trials). Yet, particularly regarding TCS, it was interesting to note that, contrasting the trend generally observed when fish are chronically exposed to xenobiotics (Diniz et al., 2009; Sadekarpawar and Parikh, 2013), the co-exposure to warming and acidification decreased fish HSI (in relation to the reference abiotic conditions; **Table 7.2.**; see also **Chapter 5. Parts 1.** and **2.**). This indicates that such stress interaction must have been too severe for the model species used in this trial (i.e. *D. sargus*), causing cellular (hepatocyte) death (Triebskorn et al., 2004; Pandey et al., 2017). Focusing on DCF's trial, in which some haematological parameters were also investigated given its anti-inflammatory mode of action, once again the significant differences observed in the total number of erythrocyte nuclear abnormalities (ENAs) were not associated with climate change effects, but rather with the exposure to DCF (**Table 7.2.;** see also **Chapter 3. Part 1.**), therefore, evidencing the genotoxicity potential of this compound. On the other hand, DCF's cytotoxicity to fish (assessed through erythrocytes' viability) seemed to have been enhanced by acidification, regardless of temperature conditions. As previously mentioned, during the trial focused on the antidepressant VFX, a parallel behavioural study was also carried out, given the psychotropic mode of action of this compound (see **Chapter 4. Part 3.**).

Table 7.2. Average percentage (%) of change of the selected whole organism endpoints in contaminated fish under climate change scenarios in relation to the reference temperature and pCO_2 levels. ↑ represents a significant increase in relation to the corresponding contaminated treatment simulating the reference temperature and pCO_2 levels, whereas ↓ indicates a significant decrease. Abbreviations: MeHg, methylmercury; DCF, diclofenac; VFX, venlafaxine; TCS, triclosan; K, Fulton's condition index; HSI, hepatosomatic index; BB_{ratio}, brain to body mass ratio; Ery:Leu, total erythrocytes to total leukocytes ratio; ENAs, erythrocyte nuclear abnormalities; Ery viable, erythrocytes' viability; nd, not determined; not significantly different (see statistical results in **Chapters 2.-6.**).

		Animal condition			**Haematological parameters**			**Animal behaviour**					
								Anxiety / Boldness		***Activity***	***Social interaction***		***Lateralization***
		K	HSI	BB_{ratio}	Ery:Leu	ENAs	Ery viable	Time to cross up	Time spent up		Time to join shoal	Time spent within shoal	
MeHg	**Warming**	ns	ns	ns	nd	nd					nd		
	Acidification	nd	nd	nd									
	Warming+ Acidification	nd	nd	nd									
DCF	**Warming**	**↓9.1**	ns	ns	ns	ns	ns				nd		
	Acidification	ns	**↑>100.0**	ns	ns	ns	**↓16.6**						
	Warming+ Acidification	**↓6.4**	ns	ns	ns	ns	**↓31.0**						
VFX	**Warming**	ns	ns	ns	nd	nd		ns	**↑>100.0**	ns	ns	**↑94.8**	**↓40.0**
	Acidification	ns	ns	ns				**↑>100.0**	**↓89.3**	ns	ns	**↑>100.0**	**↑50.0**
	Warming+ Acidification	ns	ns	ns				**↑>100.0**	**↓54.4**	**↑>100.0**	**↑>100.0**	**↑>100.0**	**↓67.0**
TCS	**Warming**	ns	ns	ns	nd	nd					nd		
	Acidification	ns	ns	ns									
	Warming+ Acidification	ns	**↓64.5**	ns									

One of the main findings in the VFX trial was that, despite all stressors affected fish behaviour to some extent, overall the most notorious behavioural alterations were found in fish co-exposed to acidification (i.e. increased animal anxiety/decreased boldness which, in turn, translated into altered activity and social interaction, as well as loss or reversion of lateralization in relation to VFX contaminated fish exposed to the reference abiotic conditions), regardless of temperature or even VFX co-exposure (**Table 7.2.**; see also **Chapter 4. Part 3.**). These changes were likely due to an impairment of fish neurotransmission mechanisms promoted by reduced seawater pH levels (Nilsson et al 2012; Hamilton et al., 2014; Munday et al., 2014), which may represent major ecological implications, potentially translating into disrupted population dynamics, as well as lower reproduction success and feeding opportunities (Pitcher and Parrish, 1993; Bisazza and Brown, 2011; Maximino et al., 2012).

As for the biochemical alterations, firstly, data highlighted the importance of evaluating the ecotoxicological responses to stressors in different fish tissues, which not only have distinct baseline biomarker levels, but also respond differently to environmental stressors, including their interactions. In fact, it was particularly interesting to verify that each studied ECC elicited differential tissue biomarker responses, therefore, evidencing their distinct modes of action in fish species. In agreement with previous findings (e.g. Islas-Flores et al., 2013; Madeira et al., 2016a,b; Rosa et al., 2016; Sampaio et al., 2018), both up- and down biomarker regulations were observed, depending on the stressor and tissue (**Table 7.3.** and **Chapters 2.-3.**). The occurrence of these contrasting strategies was linked to the fact that the exposure to environmental stressors can either: i) activate cells' defence and scavenging mechanisms in order to overcome/adjust the stress induced (e.g. increased glutathione S-transferases, GST, activity and heat shock proteins HSP70/HSC70 synthesis in fish brain co-exposed to DCF and climate change effects; increased superoxide dismutase, SOD, activity and ubiquitin, Ub, synthesis in the liver of fish co-exposed to TCS and acidification; **Table 7.3.**); or ii) inhibit these cellular mechanisms due to their exhaustion, particularly when stress conditions are too severe (in terms of intensity and duration) and exceed the thresholds of physiological tolerance of the organism/tissue (e.g. catalase, CAT, and GST inhibition in fish brain under MeHg and warming co-exposure; CAT and GST activities, as well as Ub synthesis, in the liver of fish co-exposed to VFX and acidification; **Table 7.3.**; Ferreira et al., 2015; Madeira et al., 2016a,b).

In some cases (e.g. in the muscle and/or gills of fish exposed to DCF and VFX, as well as in the liver of fish co-exposed to TCS and acidification), a significant increase of fish tissues' lipid peroxidation (LPO) was registered under the co-exposure to ECCs and abiotic stressors (in relation to the reference temperature and pCO_2 conditions; **Table 7.3.**; see also **Chapters 2.-5.)**, thus, indicating that cells' antioxidant machinery was only able to prevent to some extent the tissue damage caused by the exacerbated formation of free radicals (Madeira et al., 2013; Gonzalez-Rey and Bebianno, 2014).

Table 7.3. Average percentage (%) of change of the selected tissue specific biochemical endpoints in contaminated fish under climate change scenarios in relation to the reference temperature and pCO_2 levels. ↑ represents a significant increase in relation to the corresponding contaminated treatment simulating the reference temperature and pCO_2 levels, whereas ↓ indicates a significant decrease.

		Oxidative stress															
		CAT				**SOD**				**GST**				**LPO**			
		Muscle	*Gills*	*Liver*	*Brain*	*Muscle*	*Gills*	*Liver*	*Brain*	*Muscle*	*Gills*	*Liver*	*Brain*	*Muscle*	*Gills*	*Liver*	*Brain*
MeHg	**Warming**	ns	nd	ns	**↓53.3**	ns	nd	ns	ns	**↓66.4**	nd	**↓27.4**	**↓23.2**				
	Acidification		nd				nd				nd				nd		
	Warming+ Acidification		nd				nd				nd						
DCF	**Warming**	ns	nd	ns	ns	ns	nd	**↓10.4**	ns	**↑90.2**	nd	**↑25.0**	**↑39.3**	**↑84.7**	nd	ns	**↓15.6**
	Acidification	ns	nd	**↓32.2**	ns	**↑12.7**	nd	ns	**↑7.2**	ns	nd	ns	**↑70.9**	ns	nd	ns	**↓10.6**
	Warming+ Acidification	ns	nd	**↓21.6**	ns	ns	nd	**↑14.7**	ns	ns	nd	ns	**↑>100.0**	ns	nd	ns	**↓24.8**
VFX	**Warming**	ns	**↑>100.0**	**↓54.1**	ns	ns	ns	ns	ns	**↑11.1**	**↓71.1**	**↑20.2**	ns	ns	**↑>100.0**	ns	ns
	Acidification	ns	ns	**↓47.4**	ns	ns	ns	**↑12.8**	ns	ns	**↓26.8**	**↓41.5**	**↑48.2**	**↑>100**	**↑>100.0**	ns	ns
	Warming+ Acidification	**↑97.0**	**↑>100.0**	**↓56.9**	ns	ns	ns	**↑8.9**	**↓23.0**	**↑16.7**	ns	ns	ns	**↑>100.0**	**↑>100.0**	ns	ns
TCS	**Warming**	**↓37.9**	nd	ns	ns	ns	nd	ns	ns	ns	nd	**↓62.2**	ns	ns	nd	**↓53.5**	ns
	Acidification	ns	nd	ns	ns	ns	nd	**↑17.6**	ns	ns	nd	**↑83.1**	ns	ns	nd	**↑>100.0**	ns
	Warming+ Acidification	**↓40.3**	nd	ns	ns	ns	nd	ns	ns	ns	nd	ns	ns	ns	nd	**↓67.0**	ns

Table 7.3. ***(continuation)*** Average percentage (%) of change of the selected tissue specific endpoints in contaminated fish under climate change scenarios in relation to the reference temperature and pCO_2 levels. ↑ represents a significant increase in relation to the corresponding contaminated treatment simulating the reference temperature and pCO_2 levels, whereas ↓ indicates a significant decrease. Abbreviations: MeHg, methylmercury; DCF, diclofenac; VFX, venlafaxine; TCS, triclosan; HSP70/HSC70, heat shock protein 70 kDa content; Ub, ubiquitin content; AChE, acetylcholinesterase activity; VTG, vitellogenin content; nd, not determined; not significantly different (see statistical results in **Chapters 2.-6.**).

		Protein chaperoning and degradation								**Neurotoxicity**	**Endocrine disruption**
		HSP70/HSC70				**Ub**				**AChE**	**VTG**
		Muscle	*Gills*	*Liver*	*Brain*	*Muscle*	*Gills*	*Liver*	*Brain*	*Brain*	*Liver*
MeHg	**Warming**	ns	ns	nd	ns	nd				ns	nd
	Acidification	nd								nd	
	Warming+ Acidification	nd								nd	
DCF	**Warming**	**↑>100.0**	nd	ns	**↑>100.0**	ns	nd	**↓44.6**	ns	**↓26.1**	**↓35.8**
	Acidification	**↑77.8**	nd	ns	**↑41.5**	**↑34.9**	nd	**↓47.6**	ns	**↓15.3**	**↓50.9**
	Warming+ Acidification	**↑55.6**	nd	ns	ns	**↓35.7**	nd	**↓32.3**	ns	ns	**↓37.1**
VFX	**Warming**	ns	ns	**↓56.7**	ns	ns	**↓53.0**	**↓47.3**	ns	ns	ns
	Acidification	ns	ns	ns	ns	ns	ns	**↓62.3**	ns	**↑38.4**	**↓51.4**
	Warming+ Acidification	ns	ns	**↓75.3**	**↑85.7**	**↓30.6**	**↓49.9**	ns	ns	ns	**↓44.6**
TCS	**Warming**	**↑64.5**	nd	ns	ns	ns	nd	ns	ns	**↑17.3**	ns
	Acidification	ns	nd	**↓70.0**	ns	ns	nd	**↑>100.0**	ns	**↑27.0**	ns
	Warming+ Acidification	**↑>100.0**	nd	**↓59.3**	ns	ns	nd	**↑>100.0**	ns	ns	ns

Conversely, the opposite trend was observed in other occasions (i.e. significantly lower LPO in the brain and liver of fish exposed to DCF and TCS, respectively; **Table 7.3.**; see also **Chapters 3. Part 1.** and **4. Part 2.**), probably as a result of the enhanced antioxidant activity (i.e. CAT, SOD and/or GST activities) under increased temperature and pCO_2 levels.

Protein chaperoning and ubiquitination also constitute important cellular defence mechanisms, amending the protein damage that antioxidant scavengers were not able to prevent in the first place, with the first mechanism being responsible for repairing reversible protein damage, while the second is activated in order to signal and eliminate irreversible protein anomalies (i.e. molecular chaperones no longer can repair the cellular damage; Jackson and Durocher, 2013; Madeira et al., 2017; Sottile and Nadin, 2018). As such, the enhancement of chaperones (e.g. HSP70/HSC70) and Ub synthesis can likely indicate that increased cell damage is taking place in fish tissues (e.g. muscle of fish co-exposed to abiotic stressors and DCF or TCS; **Table 7.3.**; see also **Chapters 3. Part 1.** and **5. Parts 1.** and **2.**). On the other hand, since protein synthesis is, in general, an extremely energy-demanding process, the inhibition of protein chaperoning and ubiquitination (as observed, for instance, in the liver of fish co-exposed to abiotic stressors and DCF or VFX; **Table 7.3.**; see also **Chapters 3. Part 1.** and **4. Part 2.**) can also occur under severe stress conditions, due to impaired animal metabolism/aerobic scope (Hofmann and Somero, 1995; Gravel and Vijayan, 2007; Araújo et al., 2018).

Although changes in acetylcholinesterase (AChE) brain activity were primarily expected to occur in fish exposed to MeHg and VFX (given their well-known modes of action; Korbas et al., 2012; Bisesi et al., 2014) regardless of the surrounding abiotic conditions, the present results revealed that dietary exposure to DCF or TCS also affected marine fish neurological function (**Table 7.3.**; see also **Chapters 2.-5.**). As observed in the trials focused on MeHg and TCS (see **Chapters 3. Part 2.** and **5. Parts 1.** and **2.**), several studies have previously reported an inhibition of AChE activity following the exposure to xenobiotics, as this enzyme can be a target for many chemical compounds (Schmidel et al., 2014; Topal et al., 2017). On the other hand, and matching the results obtained in fish exposed to DCF and VFX (see **Chapters 3. Part 1.** and **3. Part 2.**), an activation of this enzyme has also been previously reported, though less commonly, and some potential justifications for this include: i) increased brain cell apoptosis, causing the release of AChE from brain cells (Zhang et al., 2002; Gonzalez-Rey and Bebianno, 2013, 2014); ii) increased synthesis of AChE splicing variants (e.g. AChE-R) under stressful conditions (Lionetto et al., 2013); or iii) disturbances of fish hypothalamo–pituitary–gonadal (HPG) axis due to xenobiotics' exposure, which enhance the synthesis of estrogens and vitellogenin (VTG)-like proteins that, in turn, modulate the cholinergic system, including AChE activity (van der Ven et al., 2006; Gonzalez-Rey and Bebianno, 2014; Oliveira et al., 2015). Although very little is known so far concerning the effects of ECCs on fish neuroendocrine system (nor the present data allows

to draw appropriate conclusions on this matter), it was interesting to see that, indeed, the increased AChE brain activity in fish exposed to DCF and VFX was accompanied by increased VTG in liver content (see **Chapters 3. Part 1** and **4. Part 2.**), whereas a reduction of both AChE activity and VTG content was found in fish exposed to TCS (see **Chapter 5. Parts 1** and **2.**; VTG content was not determined in fish exposed to MeHg). In parallel, seawater abiotic conditions, such as temperature and pH, can have a determinant role on marine biota's reproduction (namely, specimens' maturation and spawning; Brown et al., 2006; Arantes et al., 2011; Milazzo et al., 2016) and, thus, warming and acidification can likely affect fish neuroendocrine functioning, potentiating the effects elicited by the exposure to ECCs (as observed in AChE activity, when VFX was co-exposed with acidification or TCS co-exposed with acidification and warming), or reversing them (as occurred in AChE activity of fish co-exposed to abiotic stressors and DCF, as well as VTG content of fish co-exposed to abiotic stressors and DCF or VFX; **Table 7.3.**; see also **Chapters 2.-5.**). While warmer temperatures have been previously associated with increased AChE activity and VTG content, most likely due to the enhancement of fish metabolic rates (Chandra et al., 2012; Shappell et al., 2018), information regarding the effects of acidification on fish neuroendocrine system is still limited (Kwong et al., 2014; Heuer et al., 2016). In this way, the results presented in this PhD thesis constitute an important contribution to the current state of the art, evidencing that increased pCO_2 levels may have an anti-estrogenic action (i.e. VTG inhibition) or even exacerbate the effects of ECCs with anti-estrogenic modes of action in marine fish, which subsequently translate in the modulation of their cholinergic system. Such action may be attributed to the disturbance of brain ionic homeostasis under increased pCO_2 conditions which, in turn, impair fish neurotransmission and hormone synthesis (Pankhurst and Munday, 2011; Nilsson et al., 2012; Kwong et al., 2014; Heuer et al., 2016).

To sum up, the changes observed in terms of the whole animal fitness (including animal behaviour) and tissue biochemical responses revealed that the co-exposure to climate change-related stressors will certainly defy the resilience of marine organisms, particularly those inhabiting strongly polluted environments (e.g. coastal areas and estuaries). Moreover, by compromising species' welfare, recruitment and ecological success, climate change effects will also represent great challenges to the sustainability of fisheries and aquaculture sectors, therefore, calling for urgent regulatory, mitigation and/or adaptive actions at a global scale.

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CHAPTER 8.

FINAL REMARKS AND FUTURE DIRECTIONS

Final Remarks and Future Directions

The present PhD thesis provides a valuable contribution towards the understanding of climate change impacts from different scientific perpectives (e.g. ecophysiology, behavioural ecology, ecotoxicology, seafood safety), evidencing that the rising of seawater temperature and pCO_2 levels foreseen for the coming 50-100 years will likely strongly affect marine organisms' ability to cope with the presence of emerging chemical contaminants (ECCs). By affecting ECCs' bioaccumulation and detoxification mechanisms, as well as the ecotoxicological responses to these compounds, climate change effects will certainly represent great challenges to the resilience of marine invertebrate and vertebrate species, therefore, implying strong and negative impacts on the fisheries and aquaculture sectors.

The empirical findings gathered in this PhD dissertation point out to the urgent need to develop region-specific mitigation and/or adaptive strategies that will assure the sustainability of tomorrow's ocean. Hence, upon detecting climate change potential risks, which was the core of the present thesis, it is outmost crucial to clearly identify the opportunities that will emerge from it, and those include, among others: i) replacing heavily polluting technologies for "greener" and/or cleaner alternatives; ii) applying stronger efforts/pressures on policy making institutions, in order to regulate the environmental presence of ECCs, as well as GHG emissions in a tighter and more effective way; iii) developing more cost-effective wastewater treatment or even remediation (e.g. phycoremediation) technologies; iv) replacing seafood species commonly caught in the wild or farmed by other species that are more resilient to the prevailing environmental conditions and/or less ecologically threatened; v) increasing/extending the number of protected areas and/or improving their environmental management, paying particular attention to vulnerable ecosystems, such as coastal areas and estuaries that are frequently exposed to strong abiotic variations (e.g. due to their poorer hydrodynamic activity) and inputs of ECCs (e.g. due to their location near intensely urbanized regions); and, most importantly, vi) growing the ecological awareness of the general population through education, particularly in what concerns the causes and impacts of both climate change and ECCs, as well as the daily actions that can be undertaken individually to reduce the "human footprint" on the planet.

From the scientific point of view, while analysing and interpreting results in an integrated way, it also became evident that further studies are also required in the future, to support and/or complement the information presented in this PhD thesis. In this way, the trials focused on pharmaceuticals and personal care products (PPCPs), particularly the one on diclofenac (DCF; see **Chapter 3.**), highlighted the importance of further assessing the mechanisms of ECCs' biotransformation, as this presently constitutes one of literature's greatest gaps. Such knowledge will allow to better understand the toxicokinetics of ECCs in a climate change context, particularly those that can easily alternate between molecular forms according to the surrounding abiotic

conditions and/or are readily metabolized (i.e. the parental compound has a relatively short half-life, within the hour or few days' time scale) by marine biota, being transformed into different metabolites for which the toxicological attributes are still unknown. In line with this, a topic that also showed to deserve further attention was the differential effects of ECCs' exposure route. Although this matter was somewhat outside the framework of this PhD research work, during the trial focused on VFX (**Chapter 4.**) it became clear that, indeed, climate change effects can trigger distinct interactive effects (at the bioaccumulation, biochemical and behavioural levels), depending on the pathway of exposure to this compound (i.e. via water or via feed) and the tissue burdens that are subsequently reached in marine organisms. Moreover, since in the environment marine species are rarely exposed to a single ECC, but rather to a panoply of compounds, and based on the current state of the art on this matter, future ecotoxicological studies should also focus on climate change interactive effects with environmentally relevant (in terms of profile, exposure concentration and pathway) mixtures of ECCs (i.e. a topic that was only lightly tackled in **Chapter 6.**).

This PhD thesis also brought to light three research opportunities which will increase the present knowledge on climate change-related ecotoxicological impacts. Firstly, in **Chapter 3.** (i.e. the trial focused on DCF), results showed that hostile abiotic conditions can compromise fish immune system, with the co-exposure to ECCs resulting in either a stimulation or suppression of such effects. In this way, further studies on this direction (i.e. effects of environmental stressors on fish immunological responses) will be crucial in the future as one of the most expected impacts of climate change is the increased distribution, frequency of occurrence and severity of diseases (bacterioses, viroses and parasitoses) in the marine environment. These events will certainly pose a serious threat to the resilience of wild and farmed stocks and, thus, to the sustainability of fisheries and aquaculture sectors. Secondly, the way ECCs and abiotic stressors (namely, seawater pCO_2 levels) interfere with the normal functioning of fish neuroendocrine system is also a poorly studied topic, though it proved to be extremely relevant throughout **Chapters 2.-5**. As such, further research efforts should be undertaken in order to understand how and the extent to which environmental stressors can disturb fish neurotransmission and the normal course of the hypothalamo–pituitary–gonadal (HPG) axis, as these biochemical alterations can translate into impaired animal behaviour, as well as sexual differentiation and maturation, mating, spawning, hatching and territory establishment events. All these impairements can seriously compromise the dynamics at the whole population level and, ultimately, species ecological success. Thirdly, studies using species from different taxonomic groups, and from different geographical areas (tropical and temperate regions where species are able to show distinct plasticity to abiotic variations), as well as incorporating innovative "omic" tools (genomics, proteomics, transcriptomics and metabolomics) will also constitute a major research challenge in the future.

Such approaches will enable the prediction of climate change impacts from an evolutionary perspective (i.e. at the population and community levels), unveiling in depth the molecular pathways involved in animal stress responses, the potential mechanisms of genomic/phenotypic adaptation in marine species, as well as the genetic transfer of these adaptive responses across generations.

As a final consideration, and based on the distinct trends observed according to the ECC and/or climate change scenarios (i.e. when seawater warming and acidification act in isolation or combined), future research should address specific regional trends, considering not only the two studied climate change-related stressors, but also other outmost relevant climate variables, such as alterated salinity, dissolved oxygen and UV-radiation, among others. This will be crucial to the development of "tailor-made" mitigation/adaptive solutions, since climate change-related stressors and pollution (profiles and burdens) will certainly not affect marine ecosystems in the same way across the planet.

ANNEXES

ANNEX 1.

THESIS SCIENTIFIC OUTPUTS

Scientific publications in peer-reviewed journals

1. **Maulvault, A.L.**, Custódio, C., Anacleto, P., Repolho, T., Pousão, P., Nunes, M.L., Diniz, M., Rosa, R., Marques, A., 2016. Bioaccumulation and elimination of mercury in juvenile seabass (*Dicentrarchus labrax*) in a warmer environment. *Environmental Research*, 149, 77–85.

2. **Maulvault**, **A.L.**, Barbosa, V., Alves, R., Custódio, A., Anacleto, A., Repolho, T., Pousão Ferreira, P., Rosa, R., Marques, A., Diniz, M., 2017. Ecophysiological responses of juvenile seabass (*Dicentrarchus labrax*) exposed to increased temperature and dietary methylmercury. *Science of the Total Environment*, 586, 551–558.

3. **Maulvault, A.L**., Camacho, C., Barbosa, V., Alves, R., Anacleto, P., Fogaça, F., Kwadijk, C., Kotterman, M., Cunha, S.C., Fernandes, J.O., Rasmussen, R.R., Sloth, J.J., Aznar-Alemany, O., Eljarrat, E., Barceló, D., Marques, A., 2018. Assessing the effects of seawater temperature and pH on the bioaccumulation of emerging chemical contaminants in marine bivalves. *Environmental Research*, 161, 236–247.

4. **Maulvault, A.L.**, Santos, L.H.M.L.M. Santos, Paula, J.R., Camacho, C., Pissarra, V. Fogaça, F., Barbosa, V. Alves, R., Pousão Ferreira, P., Barceló, D., Rodriguez-Mozaz, S., Marques, A., Diniz, M., Rosa, R., 2018. Differential behavioural responses to venlafaxine exposure route, warming and acidification in juvenile fish (*Argyrosomus regius*). *Science of the Total Environment*, 634, 1136–1147.

5. **Maulvault, A.L.**, Santos, L.H.M.L.M. Santos, Camacho, C., Anacleto, P., Barbosa, V. Alves, R., Pousão Ferreira, P., Serra-Compte, A., Barceló, D., Rodriguez-Mozaz, S., Rosa, R., Diniz, M., Marques, A., 2018. Antidepressants in a changing ocean: Venlafaxine uptake and elimination in juvenile fish (*Argyrosomus regius*) exposed to warming and acidification conditions. *Chemosphere*, 209, 286–297.

6. **Maulvault A.L.**, Barbosa V., Alves, R., Anacleto P., Camacho C., Cunha, S., Fernandes, J.O., Pousão Ferreira, P., Rosa, R., Marques, A., Diniz, M., 2018. Integrated multi-biomarker responses of juvenile seabass to diclofenac, warming and acidification co-exposure. *Aquatic Toxicology*, 202, 65–79.

7. **Maulvault, A.L.**, Camacho C., Barbosa V., Alves, R., Anacleto P., Pousão Ferreira, P., Rosa, R., Marques, A., Diniz, M., 2019. 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*, 169, 7–25.

8. **Maulvault, A.L.**, Camacho, C., Barbosa, V., Alves, R., Anacleto, P., Cunha, S.C., Fernandes, J.O., Pousão Ferreira, P., Paula, J.R., Rosa, R., Marques, A., Diniz, M., 2019. Bioaccumulation and ecotoxicological responses of juvenile white seabream (*Diplodus sargus*) exposed to triclosan, warming and acidification. *Environmental Pollution*, 245, 427–442.

Book chapter

Maulvault, A.L., Anacleto, P., Marques, A., Diniz, M., Rosa, R. (in press). Chemical contaminants in a changing ocean. In: B. Duarte, I. Caçador (eds.). Ecotoxicology of marine organisms. CRC Press. 375 pp (ISBN: 1138035491).

Oral presentations at national and international conferences

1. Maulvault, A.L., Anacleto, P., Repolho, T., Pousão, P., Nunes, M.LRosa, R., Marques, A., 2014. Effect of warming on bioaccumulation, speciation and elimination of mercury in juvenile seabass (*Dicentrarchus labrax*). Aquaculture 2014, Bilbao, Spain.

2. Maulvault, A.L., Anacleto, P., Lopes, A.R., Repolho, T., Pousão, P., Nunes, M.L., Diniz, M., Rosa, R., Marques, A., 2015. Coping with mercury in a warmer ocean: Tissue partitioning and ecophysiological implications in seabass (*Dicentrarchus labrax*). ICFC 2015, Lisbon, Portugal.

3. Maulvault, A.L., Anacleto, P., Lopes, A.R., Repolho, T., Pousão, P., Nunes, M.L., Diniz, M., Rosa, R., Marques, A., 2015. Coping with mercury in a warmer ocean: Tissue partitioning, speciation, and ecophysiological implications in seabass (*Dicentrarchus labrax*). SETAC 2015, Barcelona, Spain.

4. Maulvault, A.L., Sampaio, E., Cunha, S., Barbosa, V., Alves, R., Ferreira-Pousão, P., Rosa, R., Diniz, M., Marques, A., 2016. Coping with pharmaceuticals and personal care products (PPCP) in a changing ocean: Bioaccumulation and ecophysiological implications in seabass (*Dicentrarchus labrax*) exposed to diclofenac. SETAC 2016, Nantes, France.

5. Maulvault, A.L., Paula, J.R., Pissarra, V., Barbosa, V., Fogaça, F. Alves, R., Ferreira-Pousão, P., Diniz, M., Marques, A. Rosa, R., 2016. Coping with antidepressants in a changing ocean: Behavioural implications in juvenile meagre exposed to venlafaxine. XIII SPE 2016, Lisbon, Portugal.

6. Maulvault, A.L., Camacho, C., Sampaio, E., Barbosa, V., Alves, R., Fogaça, F., Kwadijke, C., Kotterman, M., Cunha, S., Fernandes, J., Rasmussen, R.R., Sloth, J.J., Aznar-Alemany, O., Eljarrath, E., Barceló, D., Marques, A., 2017. Can seafood safety be compromised in the ocean of tomorrow?. International stakeholder event & open science conference – Seafood Safety New Findings & Innovation Challenges, 2017, Brussels, Belgium.

7. Maulvault, A.L., Santos, L.H., Paula, J.R., Barbosa, V., Alves, R., Anacleto, P., Rodriguez-Mozaz, S., Pousão Ferreira, P., Diniz, M., Rosa, R. Marques, A., 2018. Coping with antidepressants in a changing ocean: Tissue bioaccumulation and behavioural implications in juvenile *Argyrosomus regius* exposed to venlafaxine. Setac 2018, Rome, Italy.

ANNEX 2.

SUPPLEMENTARY INFORMATION FOR CHAPTER 2. PART 1.

Bioaccumulation and elimination of mercury in juvenile seabass (Dicentrarchus labrax) in a warmer environment

TABLES

Table A.2.1. Composition of control and MeHg-enriched diets (i.e. dry inert pellets) used for juvenile seabass feeding.

Ingredients	%DW
Fishmeal LT70 [1]	28
Fishmeal 60 [2]	20
Fish soluble concentrate [3]	2.5
Soy protein concentrate [4]	5
Wheat gluten [5]	5.5
Maize gluten [6]	5
Soybean meal 48 [7]	9
Wheat meal	5.5
Whole peas	5
Fish oil [8]	13.5
Vitamin and mineral premix [9]	1
Dry matter (DM), %	94.3
Crude protein, %DM	51.7
Crude fat, %DM	18.9
Ash, %DM	9.6

[1] Peruvian fishmeal LT: 71% crude protein (CP), 11% crude fat (CF) (EXALMAR, Peru); [2] Fair Average Quality (FAQ) fishmeal: 62% CP, 12%CF (COFACO, Portugal); [3]CPSP 90: 84% CP, 12% CF (Sopropêche, France); [4] Soycomil P: 65% CP, 0.8% CF (ADM, The Netherlands); [5] VITEN: 85.7% CP, 1.3% CF (ROQUETTE, France); [6] Maize gluten feed: 61% CP, 6% CF (COPAM, Portugal); [7] Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF (SORGAL SA, Portugal); [8] Fish oil (COPPENS International, The Netherlands); [9] Premix for marine fish (PREMIX Lda, Portugal). Vitamins (IU or mg kg^{-1} diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg kg^{-1} diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middlings.

Table A.2.2. Concentration of MeHg in feeds (dry weight) and T-Hg in seawater sampled from the four treatments during the trial (mean ± standard deviation mg kg^{-1}; n = 3). Abbreviations: 18_control – seawater temperature set at 18 °C and animals fed with control diet; 18_MeHg-enriched – seawater temperature set at 18 °C and animals fed with MeHg-enriched diet; 22_control – seawater temperature set at 22 °C and animals fed with control diet; 22_MeHg-enriched – seawater temperature set at 22 °C and animals fed with MeHg-enriched diet.

Feed		Water			
control	MeHg-enriched	18_control	18_MeHg-enriched	22_control	22_MeHg-enriched
0.60 ± 0.02	8.12 ± 0.07	< 0.005	< 0.005	< 0.005	< 0.005
0.59 ± 0.03	8.02 ± 0.05	< 0.005	< 0.005	< 0.005	< 0.005
0.61 ± 0.01	7.81 ± 0.05	< 0.005	< 0.005	< 0.005	< 0.005
0.62 ± 0.01	8.10 ± 0.07	< 0.005	< 0.005	< 0.005	< 0.005
0.61 ± 0.02	7.97 ± 0.04	< 0.005	< 0.005	< 0.005	< 0.005
0.59 ± 0.01	7.92 ± 0.06	< 0.005	< 0.005	< 0.005	< 0.005
0.60 ± 0.01	8.22 ± 0.05	< 0.005	< 0.005	< 0.005	< 0.005

Table A.2.3. Moisture content (%) in three tissues (muscle, liver and brain) of juvenile seabass (mean ± standard deviation; n = 6) sampled in each treatment, during 28 days of exposure to MeHg and 28 days of depuration. Abbreviations: 18_control – seawater temperature set at 18 °C and animals fed with control diet; 18_MeHg-enriched – seawater temperature set at 18 °C and animals fed with MeHg-enriched diet; 22_control – seawater temperature set at 22 °C and animals fed with control diet; 22_MeHg-enriched – seawater temperature set at 22 °C and animals fed with MeHg-enriched diet.

		18_control	22_control	18_MeHg-enriched	22_MeHg-enriched
	Muscle	74.3 ± 1.8	74.9 ± 2.0	74.7 ± 2.2	73.3 ± 1.9
	Liver	59.4 ± 5.2	63.0 ± 3.4	63.9 ± 3.7	60.5 ± 3.6
0	Brain	78.0 ± 2.1	77.2 ± 3.2	76.9 ± 2.0	76.2 ± 2.7
	Muscle	72.4 ± 2.2	73.9 ± 1.6	73.1 ± 2.5	74.5 ± 2.3
	Liver	60.2 ± 3.3	63.4 ± 4.2	63.7 ± 4.7	63.3 ± 5.0
7	Brain	76.3 ± 3.3	76.2 ± 2.4	75.8 ± 3.6	77.3 ± 3.3
	Muscle	71.6 ± 1.7	72.7 ± 1.5	73.2 ± 2.4	73.9 ± 1.7
	Liver	62.3 ± 4.3	58.7 ± 5.1	58.9 ± 5.3	58.8 ± 4.8
14	Brain	77.1 ± 2.7	75.9 ± 4.1	75.9 ± 2.9	77.6 ± 2.8
	Muscle	73.1 ± 1.4	72.6 ± 1.9	74.1 ± 2.7	72.6 ± 1.5
	Liver	58.9 ± 5.1	59.9 ± 4.8	60.9 ± 2.7	57.6 ± 4.7
28	Brain	78.4 ± 4.3	76.7 ± 3.2	77.4 ± 3.5	76.7 ± 3.0
	Muscle	73.5 ± 2.3	73.3 ± 2.1	73.8 ± 1.8	73.9 ± 1.8
	Liver	62.1 ± 2.1	62.5 ± 3.9	61.3 ± 3.7	62.4 ± 3.4
35	Brain	75.2 ± 3.6	78.2 ± 2.5	76.2 ± 3.2	78.2 ± 1.9
	Muscle	73.4 ± 2	74.0 ± 2.0	74.0 ± 2.1	74.2 ± 1.4
	Liver	61.5 ± 2.6	61.4 ± 4.6	61.0 ± 4.9	63.7 ± 3.9
42	Brain	75.3 ± 3.0	75.4 ± 3.1	76.5 ± 2.7	76.3 ± 2.4
	Muscle	74.1 ± 1.7	74.2 ± 1.8	73.4 ± 1.6	73.2 ± 2.2
	Liver	63.0 ± 2.4	61.4 ± 3.7	59.9 ± 3.2	62.6 ± 4.5
56	Brain	77.2 ± 1.6	76.8 ± 2.6	77.6 ± 3.1	75.4 ± 3.3

Table A.2.4. Total Hg content (mg kg^{-1} of wet weight) in three tissues (muscle, liver and brain) of juvenile seabass (mean ± standard deviation; n = 6) sampled in each of the four treatments, during 28 days of exposure to MeHg and 28 days of depuration. Abbreviations: 18_control – seawater temperature set at 18 °C and animals fed with control diet; 18_MeHg-enriched – seawater temperature set at 18 °C and animals fed with MeHg-enriched diet; 22_control – seawater temperature set at 22 °C and animals fed with control diet; 22_MeHg-enriched – seawater temperature set at 22 °C and animals fed with MeHg-enriched diet.

		18_control	22_control	18_MeHg-enriched	22_MeHg-enriched
0	Muscle	0.27 ± 0.09	0.27 ± 0.09	0.27 ± 0.09	0.27 ± 0.09
	Liver	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
	Brain	0.17 ± 0.01	0.17 ± 0.01	0.17 ± 0.01	0.17 ± 0.01
7	Muscle	0.35 ± 0.12	0.33 ± 0.05	0.81 ± 0.27	1.13 ± 0.18
	Liver	0.07 ± 0.00	0.07 ± 0.00	0.98 ± 0.31	2.78 ± 0.27
	Brain	0.21 ± 0.00	0.21 ± 0.00	1.41 ± 0.08	1.62 ± 0.02
14	Muscle	0.28 ± 0.05	0.27 ± 0.01	1.63 ± 0.52	1.95 ± 0.26
	Liver	0.10 ± 0.00	0.10 ± 0.00	2.37 ± 0.49	2.85 ± 0.10
	Brain	0.20 ± 0.02	0.2 ± 0.02	2.15 ± 0.02	3.32 ± 0.54
28	Muscle	0.29 ± 0.01	0.29 ± 0.01	4.02 ± 0.24	4.85 ± 0.30
	Liver	0.09 ± 0.01	0.09 ± 0.02	6.05 ± 1.83	5.43 ± 1.24
	Brain	0.18 ± 0.02	0.18 ± 0.02	5.74 ± 0.06	8.6 ± 0.42
35	Muscle	0.24 ± 0.03	0.24 ± 0.02	4.63 ± 0.32	5.38 ± 0.38
	Liver	0.10 ± 0.00	0.10 ± 0.01	4.00 ± 0.18	4.67 ± 0.31
	Brain	0.19 ± 0.01	0.19 ± 0.01	7.44 ± 0.72	9.73 ± 0.94
42	Muscle	0.24 ± 0.04	0.23 ± 0.01	3.78 ± 0.29	4.58 ± 0.33
	Liver	0.06 ± 0.01	0.06 ± 0.01	2.29 ± 0.08	5.00 ± 0.60
	Brain	0.18 ± 0.02	0.18 ± 0.02	5.88 ± 0.02	7.84 ± 0.50
56	Muscle	0.25 ± 0.02	0.24 ± 0.01	3.93 ± 0.27	4.58 ± 0.21
	Liver	0.08 ± 0.01	0.08 ± 0.02	2.09 ± 0.27	3.10 ± 0.31
	Brain	0.24 ± 0.00	0.24 ± 0.00	4.86 ± 0.56	7.24 ± 0.27

ANNEX 3.

SUPPLEMENTARY INFORMATION FOR CHAPTER 2. PART 2.

Ecophysiological responses of juvenile seabass (Dicentrarchus labrax) exposed to increased temperature and dietary methylmercury

TABLES

Table A.3.1. Composition of control and MeHg-enriched diets (i.e. dry inert pellets) used for juvenile seabass feeding.

Ingredients	% dry weight
Fishmeal LT70 [1]	28
Fishmeal 60 [2]	20
Fish soluble concentrate [3]	2.5
Soy protein concentrate [4]	5.0
Wheat gluten [5]	5.5
Maize gluten [6]	5.0
Soybean meal 48 [7]	9.0
Wheat meal	5.5
Whole peas	5.0
Fish oil [8]	13.5
Vitamin and mineral premix [9]	1.0
Dry matter (DM), %	94.3
Crude protein, %DM	51.7
Crude fat, %DM	18.9
Ash, %DM	9.6

[1] Peruvian fishmeal LT: 71% crude protein (CP), 11% crude fat (CF) (EXALMAR, Peru); [2] Fair Average Quality (FAQ) fishmeal: 62% CP, 12%CF (COFACO, Portugal); [3]CPSP 90: 84% CP, 12% CF (Sopropêche, France); [4] Soycomil P: 65% CP, 0.8% CF (ADM, The Netherlands); [5] VITEN: 85.7% CP, 1.3% CF (ROQUETTE, France); [6] Maize gluten feed: 61% CP, 6% CF (COPAM, Portugal); [7] Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF (SORGAL SA, Portugal); [8] Fish oil (COPPENS International, The Netherlands); [9] Premix for marine fish (PREMIX Lda, Portugal). Vitamins (IU or mg kg^{-1} diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg kg^{-1} diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middlings.

Table A.3.2. Concentration of MeHg in feeds (dry weight) and T-Hg in seawater sampled from the four treatments (mean ± standard deviation mg kg^{-1}; n = 3), throughout the 28 days of trial. Abbreviations: 18_control – seawater temperature set at 18 °C and animals fed with control diet; 18_MeHg-enriched – seawater temperature set at 18 °C and animals fed with MeHg-enriched diet; 22_control – seawater temperature set at 22 °C and animals fed with control diet; 22_MeHg-enriched – seawater temperature set at 22 °C and animals fed with MeHg-enriched diet; LOD – limit of detection in the methodology used for THg and MeHg quantification (0.005 mg L^{-1}).

Feed		Seawater			
control	MeHg-enriched	18_control	18_MeHg-enriched	22_control	22_MeHg-enriched
0.60 ± 0.02	8.12 ± 0.07	< LOD	< LOD	< LOD	< LOD
0.59 ± 0.03	8.02 ± 0.05	< LOD	< LOD	< LOD	< LOD
0.61 ± 0.01	7.81 ± 0.05	< LOD	< LOD	< LOD	< LOD
0.62 ± 0.01	8.10 ± 0.07	< LOD	< LOD	< LOD	< LOD

ANNEX 4.

SUPPLEMENTARY INFORMATION FOR CHAPTER 3. PART 1.

Integrated multi-biomarker responses of juvenile seabass to diclofenac, warming and acidification co-exposure

TABLES

Table A.4.1. Proximate chemical composition of CRT and DCF-enriched feeds.

Ingredients	%
Fishmeal LT70[1]	20.0
Fish protein concentrate[2]	20.5
Squid meal[3]	25.0
Krill meal[4]	5.0
Wheat gluten[5]	11.0
Fish oil[6]	8.0
Soy lecithin[7]	3.5
Guar gum[8]	1.3
Macroalgae mix[9]	2.0
Antioxidant[10]	0.5
Monocalcium phosphate[11]	1.3
Vitamin and mineral premix[12]	2.0

[1] Peruvian fishmeal LT: 67% crude protein (CP), 9% crude fat (CF), EXALMAR, Peru.
[2] CPSP 90: 84% CP, 12% CF, Sopropêche, France.
[3] Super prime without guts: 82% CP, 3.5% CF, Sopropêche, France;
[4] Krill meal: 52.4% CP, 21.7% CF, Aker Biomarine, Norway.
[5] VITAL: 85.7% CP, 1.3% CF, ROQUETTE, France.
[6] SAVINOR UTS, Portugal
[7] LECICO P 700IPM, LECICO GmbH, Germany.
[8] Guar gum HV109, SEAH International, France.
[9] OceanFeed: 10.2% CP, Ocena Harvest Technology, Ireland.
[10] Paramega PX, Kemin Europe NV, Belgium
[11] Monocalcium phosphate: 22% phosphorus, 16% calcium, Fosfitalia, Italy.
[12] PREMIX Lda, Portugal: Vitamins (IU or mg kg^{-1} diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg kg^{-1} diet): copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; excipient wheat middlings.

Table A.4.2. Seawater physical and chemical parameters (mean ± standard deviation) in each treatment. Abbreviations: Control – reference temperature and pH conditions (i.e. T = 19 ºC and pH = 8.0 units); Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC); DCF – DCF exposure; TA – total alkalinity; pCO_2 - partial CO_2 pressure; TCO_2 - Total CO_2 concentration; HCO_3^- - bicarbonate; CO_3^{2-} - carbonate ion concentrations; ΩCal - calcite saturation state; ΩAra – aragonite saturation state.

	Control	Acid	DCF+Acid	Warm	DCF+Warm	Acid+Warm	DCF+Acid+Warm
Temperature (°C)	19.2 ± 0.1	19.3 ± 0.2	19.2 ± 0.1	24.3 ± 0.2	24.4 ± 0.1	24.3 ± 0.3	24.4 ± 0.1
Salinity	35.2 ± 0.3	35.0 ± 0.3	35.2 ± 0.4	35.0 ± 0.3	35.2 ± 0.3	35.0 ± 0.3	35.2 ± 0.4
pH	8.02 ± 0.05	7.62 ± 0.04	7.61 ± 0.06	7.97 ± 0.03	8.00 ± 0.04	7.58 ± 0.03	7.61 ± 0.06
TA (µmol kg^{-1})	2752.2 ± 70	2997.8 ± 98.2	2824.3 ± 46	2595.2 ± 55.4	2824.1 ± 68.8	2779 ± 110.5	2939.7 ± 221.9
***p*CO$_2$ (µatm)**	500.9 ± 65.8	1540 ± 108.5	1501.9 ± 191.8	542.7 ± 48.6	544.3 ± 53.5	1597.5 ± 59.9	1580.6 ± 135.3
***T*CO$_2$ (µmol kg^{-1})**	2241.9 ± 63.4	2752 ± 79.3	2597 ± 30.7	2088.2 ± 62.1	2243.3 ± 37.3	2529.8 ± 94.6	2659.1 ± 192.9
***HCO$_3^-$* (µmol kg^{-1})**	2241.9 ± 66.7	2752 ± 70.4	2597 ± 26.8	2088.2 ± 64.1	2243.3 ± 22.7	2529.8 ± 85.1	2659.1 ± 174.5
CO$_3^{2-}$ (µmol kg^{-1})	213.6 ± 24.3	104.4 ± 12.3	95.6 ± 12.6	210.9 ± 5.5	245 ± 26.2	105.1 ± 11.3	119.3 ± 22.3
Ω Ara	5.1 ± 0.6	2.5 ± 0.3	2.3 ± 0.3	5.1 ± 0.1	5.9 ± 0.6	2.5 ± 0.3	2.9 ± 0.5
Ω Cal	3.3 ± 0.4	1.6 ± 0.2	1.5 ± 0.2	3.3 ± 0.1	3.9 ± 0.4	1.7 ± 0.2	1.9 ± 0.4

Table A.4.3. Basal biomarker levels (mean ± SD; *n* = 9) in juvenile *D. labrax*. Abbreviations: TL - total length; W – weight; K - Fulton's condition index; HSI - hepatosomatic index; BB_{ratio} - brain-to-body mass ratio; Ery - percentage of erythrocytes in relation to total cell counts; Leu - percentage of leukocytes in relation to total cell counts; Viable Ery - percentage of viable erythrocytes in relation to total erythrocyte counts; ENAs – percentage of erythrocyte nuclear abnormalities in relation to total erythrocyte counts; Micronuclei – percentage of micronuclei in relation to total erythrocyte counts; CAT – catalase activity; SOD – superoxide dismutase inhibition; GST – glutathione S-transferase activity; LPO - lipid peroxidation; HSP70/HSC70 – heat shock proteins content; Ub – total ubiquitin content; AChE – acetylcholinesterase activity; VTG – vitellogenin content.

	Whole body		
TL (cm)	7.0 ± 0.2		
W (g)	6.7 ± 0.3		
K	1.93 ± 0.13		
HSI	2.39 ± 0.69		
BB_{ratio}	0.97 ± 0.08		
Ery (%)	94.9 ± 0.9		
Leu (%)	5.1 ± 0.9		
Viable (%)	77.4 ± 2.3		
ENAs (%)	9.7 ± 3.3		
Micronuclei (%)	0.0 ± 0.0		
	Muscle	**Liver**	**Brain**
CAT (µM min^{-1} mg^{-1} protein)	3.5 ± 1.4	5.6 ± 0.7	4.1 ± 1.0
SOD (% inhibition)	39.1 ± 1.9	71.4 ± 2.2	70.3 ± 2.9
GST (nmol min^{-1} mg^{-1} protein)	7.8 ± 0.1	29.2 ± 4.4	9.5 ± 0.4
LPO (nmol mg^{-1} protein)	0.009 ± 0.001	0.004 ± 0.001	0.014 ± 0.002
HSP70/HSC70 (µg mg^{-1} protein)	0.11 ± 0.01	0.05 ± 0.01	0.11 ± 0.01
Ub (µg mg^{-1} protein)	0.018 ± 0.001	0.009 ± 0.003	0.004 ± 0.001
AChE (nmol min^{-1} mg^{-1} protein)	-	-	36.7 ± 2.0
VTG (ng mg^{-1} protein)	-	189.9 ± 0.5	-

Table A.4.4. Summary of changes (%) induced by DCF exposure, acidification and warming in relation to the average values obtained in Control treatment. "↑" before the value indicates a significant increase compared to values found in Control treatment, whereas "↓" indicates a significant decrease ($p < 0.05$). Abbreviations: K - Fulton's condition index; HIS - hepatosomatic index; BB_{ratio} - brain-to-body mass ratio, Ery:Leu – ratio between total erythrocytes and total leukocytes counts; ENAs - erythrocytes nuclear abnormalities (including micronuclei); Viable Ery - viable erythrocytes; CAT – catalase; SOD – superoxide dismutase; GST – glutathione S-transferase; LPO - lipid peroxidation; HSP70/HSC70 - heat shock proteins; Ub - total ubiquitin; AChE – acetylcholinesterase; VTG – vitellogenin; NS – No significant alteration ($p > 0.05$) in relation to the Control treatment; DCF – DCF exposure; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC).

	Fish condition			Hematological parameters			Biomarkers								
Stressor	**K**	**HSI**	**BB_{ratio}**	**Ery:Leu**	**ENAs**	**Viable Ery**	**Tissue**	**CAT**	**SOD**	**GST**	**LPO**	**HSP70/ HSC70**	**Ub**	**AChE**	**VTG**
Acidification	NS	NS	↓ 47%	NS	NS	↓ 15%	Muscle	NS	↑ 58%	↓ 33%	NS	↓ 24%	NS	-	-
							Liver	NS	↓ 11%	↓ 24%	NS	NS	NS	-	↓ 14%
							Brain	NS	NS	NS	NS	↑ 28%	↑ >100%	NS	-
Warming	NS	NS	↓ 26%	NS	NS	NS	Muscle	NS	↑ 53%	NS	↑ 66%	NS	NS		
							Liver	NS	NS	↑ 63%	NS	↑ 56%	↑ 48%		↑ 40%
							Brain	NS	NS	↑ >100%	NS	↑ 50%	↑ 40%	NS	
Acidification + Warming	NS	NS	↓ 44%	↓ 57%	NS	↓ 11%	Muscle	NS	↑ 67%	NS	↑ >100%	NS	NS	-	-
							Liver	NS	NS	NS	NS	↑ 67%	↑ 34%	-	↑ 39%
							Brain	NS	NS	↑ 50%	NS	NS	NS	NS	-
DCF exposure	NS	↓ 55%	↓ 25%	NS	↑ >100%	↓ 37%	Muscle	↓ 80%	↑ 44%	NS	NS	NS	↑ 15%	-	-
							Liver	NS	↓ 9%	NS	NS	NS	NS	-	↑ 87%
							Brain	NS	NS	↑ 23%	NS	↓ 40%	NS	↑ 17%	-
DCF exposure + Acidification	NS	NS	↓ 30%	NS	↑ >100%	↓ 48%	Muscle	↓ 63%	↑ 25%	NS	↓ 48%	↑ 44%	↑ 55%	-	-
							Liver	NS	↓ 9%	NS	NS	NS	↓ 36%	-	NS
							Brain	NS	NS	↑ >100%	NS	NS	NS	NS	-
DCF exposure + Warming	↓ 12%	NS	↓ 29%	NS	↑ >100%	↓ 40%	Muscle	↓ 57%	↑ 36%	↑ 88%	↑ 45%	↑ 84%	NS	-	-
							Liver	NS	NS	↑ 21%	NS	NS	↓ 33%	-	↑ 20%
							Brain	NS	NS	↑ 72%	NS	↑ 23%	NS	↓ 13%	-
DCF exposure + Acidification + Warming	↓ 10%	↓ 34%	↓ 18%	NS	↑ >100%	↓ 57%	Muscle	↓ 69%	↑ 36%	NS	↓ 30%	NS	↓ 26%	-	-
							Liver	NS	NS	NS	NS	NS	↓ 18%	-	NS
							Brain	NS	NS	↑ >100%	NS	↓ 37%	NS	NS	-

Table A.4.5. Summary of IBR scores measured in each treatment, for the whole organism and different tissues. Values in bold correspond to individual biomarker scores (S) that differed in ≥ 0.5 from the score in Control treatment. Abbreviations: K - Fulton's condition index; HIS - hepatosomatic index; BB_{ratio} - brain-to-body mass ratio, Ery:Leu – ratio between total erythrocytes and total leukocytes counts; ENAs - erythrocytes nuclear abnormalities (including micronuclei); Viable Ery - viable erythrocytes; CAT – catalase; SOD – superoxide dismutase; GST – glutathione S-transferase; LPO - lipid peroxidation; HSP70/HSC70 - heat shock proteins; Ub - total ubiquitin; AChE – acetylcholinesterase; VTG – vitellogenin; DCF – DCF exposure; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC).

		K	HSI	BB_{ratio}	Ery:Leu	ENAs	Viable Ery		CAT	SOD	GST	LPO	HSP70/HSC70	Ub	AChE	VTG
								Muscle	1.67	0.32	1.30	1.06	1.59	1.76	-	-
Control	***Whole organism***	1.05	4.20	0.00	1.02	0.76	0.36	***Liver***	2.70	2.33	1.22	4.69	2.67	2.01	-	1.01
								Brain	**2.11**	**3.14**	**0.15**	**2.89**	**1.59**	**0.55**	1.85	-
								Muscle	**2.52**	**3.09**	**3.17**	**1.79**	**0.98**	**0.95**	-	-
Acid	***Whole organism***	**2.07**	**3.18**	0.37	**0.35**	0.56	**1.05**	***Liver***	**1.90**	**0.32**	**0.35**	**2.39**	2.65	2.34	-	0.60
								Brain	**3.28**	**4.10**	**2.36**	3.61	**0.75**	**3.30**	1.77	-
								Muscle	1.86	**2.81**	1.43	**2.23**	**1.06**	**2.27**	-	-
Warm	***Whole organism***	**1.94**	**3.65**	**1.61**	**1.60**	**2.63**	0.35	***Liver***	**1.71**	**1.45**	**3.54**	5.06	**4.74**	**3.56**	**-**	**2.22**
								Brain	**2.97**	**3.66**	**1.96**	**2.99**	3.17	**1.17**	**2.49**	-
								Muscle	**2.54**	**3.52**	1.55	**3.14**	**1.11**	1.31	-	-
Acid+Warm	***Whole organism***	**1.81**	**3.32**	**0.57**	**0.23**	**2.33**	**0.84**	***Liver***	2.94	**1.51**	**0.76**	4.72	**5.17**	**3.10**	-	**2.21**
								Brain	**3.94**	**3.95**	**1.57**	**3.50**	**1.48**	1.08	1.63	-
								Muscle	**0.14**	**2.39**	1.27	0.67	**1.10**	**2.34**	-	-
DCF	***Whole organism***	**1.94**	**1.12**	**1.54**	**1.76**	**2.94**	**2.03**	***Liver***	**4.39**	**0.66**	1.10	**3.74**	**3.92**	**2.71**	-	**3.66**
								Brain	**3.70**	**5.43**	**2.03**	**3.49**	**0.33**	0.53	**3.69**	-
								Muscle	**0.45**	**2.92**	1.20	**0.19**	**2.71**	**3.90**	-	-
DCF+Acid	***Whole organism***	**2.45**	**2.16**	**1.54**	**3.07**	**2.70**	**2.50**	***Liver***	2.30	**0.69**	0.83	**2.59**	2.59	**0.82**	-	**2.65**
								Brain	**3.60**	**1.86**	**2.04**	**2.32**	**1.13**	0.24	1.77	-
								Muscle	**0.57**	**2.39**	**3.75**	**1.87**	**3.73**	2.11	-	-
DCF+Warm	***Whole organism***	0.92	**2.56**	**1.61**	**2.45**	**2.60**	**2.17**	***Liver***	**4.30**	2.42	**2.00**	4.37	**3.96**	**0.94**	-	**1.79**
								Brain	**2.03**	**3.84**	**1.38**	**1.77**	**2.32**	0.42	**0.41**	-
								Muscle	**0.34**	**2.38**	**2.18**	**0.51**	**2.25**	**0.73**	-	-
DCF+Acid+Warm	***Whole organism***	**1.56**	**2.33**	**1.09**	**2.03**	**2.29**	**2.91**	***Liver***	2.99	**3.15**	1.08	**3.67**	**4.08**	**2.80**	-	**1.86**
								Brain	**4.81**	**3.68**	**3.00**	**0.75**	**0.41**	0.41	**3.08**	-

ANNEX 5.

SUPPLEMENTARY INFORMATION FOR CHAPTER 4. PART 1.

Antidepressants in a changing ocean: Venlafaxine uptake and elimination in juvenile fish (Argyrosomus regius) exposed to warming and acidification conditions

TABLES

Table A.5.1. Proximate chemical composition of CRT and VFX-enriched feeds.

	%
Fishmeal LT70[1]	28.0
Fishmeal 60[2]	20.0
Fish protein concentrate[3]	2.5
Soy protein concentrate[4]	5.0
Wheat gluten[5]	5.5
Corn gluten[6]	5.0
Soybean meal 48[7]	9.0
Wheat meal[8]	5.0
Whole peas[9]	5.0
Fish oil[10]	13.5
Guar gum[11]	0.5
Vitamin and mineral premix[12]	1.0

[1] Peruvian fishmeal LT: 67% crude protein (CP), 9% crude fat (CF), EXALMAR, Peru.
[2] Fair Average Quality (FAQ) fishmeal: 62% CP, 12% CF, COFACO, Portugal.
[3] CPSP 90: 84% CP, 12% CF, Sopropêche, France.
[4] Soycomil P: 65% CP, 0.8% CF, ADM, The Netherlands.
[5] VITAL: 85.7% CP, 1.3% CF, ROQUETTE, France.
[6] GLUTALYS: 61% CP, 6 % CF, ROQUETTE, France.
[7] Dehulled solvent extracted soybean meal: 47% CP, 2.6% CF, Cargill, Spain.
[8] Whole wheat: 10.2% CP, 1.2% CF, Casa Lanchinha, Portugal.
[9] Whole peas: 19.8% CP, 2.2% CF, Casa Lanchinha, Portugal.
[10] SAVINOR UTS, Portugal
[11] Guar gum HV109, SEAH International, France.
[12] PREMIX Lda, Portugal: Vitamins: DL-alpha tocopherol acetate, 100 mg kg^{-1}; sodium menadione bisulphate, 25 mg kg^{-1}; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg kg^{-1}; riboflavin, 30 mg kg^{-1}; pyridoxine, 20 mg kg^{-1}; cyanocobalamin, 0.1 mg kg^{-1}; nicotinic acid, 200 mg kg^{-1}; folic acid, 15 mg kg^{-1}; ascorbic acid, 500 mg kg^{-1}; inositol, 500 mg kg^{-1}; biotin, 3 mg kg^{-1}; calcium panthotenate, 100 mg kg^{-1}; choline chloride, 1000 mg kg^{-1}, betaine, 500 mg kg^{-1}. Minerals: copper sulphate, 9 mg kg^{-1}; ferric sulphate, 6 mg kg^{-1}; potassium iodide, 0.5 mg kg^{-1}; manganese oxide, 9.6 mg kg^{-1}; sodium selenite, 0.01 mg kg^{-1}; zinc sulphate,7.5 mg kg^{-1}; sodium chloride, 400 mg kg^{-1}; excipient wheat middlings.

Table A.5.2. Seawater physical and chemical parameters (mean ± standard deviation) in each treatment. Abbreviations: Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC); VFX-water – fish exposed to venlafaxine via water; VFX-feed – fish exposed to venlafaxine via feed; TA – total alkalinity; *P* CO_2 - partial CO_2 pressure; *T* CO_2 - Total CO_2 concentration; HCO_3^- - bicarbonate; CO_3^{2-} - carbonate ion concentrations; ΩCal - calcite saturation state; ΩAra – aragonite saturation state.

	Control	VFX-feed	Acid+VFX-feed	Warm+VFX-feed	Acid+Warm+VFX-feed	VFX-water	Acid+Warm+VFX-water
Temperature (°C)	19.0 ± 0.2	19.0 ± 0.1	19.0 ± 0.1	24.0 ± 0.2	24.0 ± 0.2	19.0 ± 0.3	24.0 ± 0.2
Salinity	35.2 ± 0.5	35.1 ± 0.5	35.1 ± 0.7	35.5 ± 0.6	35.3 ± 0.6	35.1 ± 0.7	35.4 ± 0.8
pH	8.02 ± 0.01	8.01 ± 0.04	7.63 ± 0.02	8.04 ± 0.03	7.62 ± 0.04	8.05 ± 0.03	7.62 ± 0.03
TA (µmol kg^{-1})	2737.1 ± 20.9	2715.4 ± 33.1	2648.0 ± 135.8	2681.2 ± 68.2	2494.5 ± 71.4	2705.9 ± 33.2	2565.5 ± 191.6
***P* CO_2 (µatm)**	540.8 ± 4.6	535.5 ± 6.8	1477.7 ± 78.4	523.2 ± 12.7	1394.8 ± 39.0	534.6 ± 8.5	1433.9 ± 107.8
***T* CO_2 (µmol kg^{-1})**	2467.9 ± 21.2	2444.9 ± 29.3	2572.1 ± 134.5	2371.5 ± 60.1	2392.9 ± 68.2	2437.4 ± 33.8	2461.4 ± 185.9
***HCO_3^-* (µmol kg^{-1})**	2242.7 ± 20.5	2220.1 ± 25.5	2434 ± 127.6	2121.9 ± 52.3	2252.4 ± 63.6	2214.0 ± 32.4	2316.5 ± 174.5
CO_3^{2-} (µmol kg^{-1})	207.3 ± 0.5	207.3 ± 3.7	89.4 ± 4.3	234.4 ± 7.5	100.4 ± 3.7	205.9 ± 1.0	103.7 ± 8.4
Ω Ara	3.2 ± 0.1	3.2 ± 0.1	1.4 ± 0.1	3.7 ± 0.1	1.6 ± 0.1	3.2 ± 0.2	1.6 ± 0.1
Ω Cal	4.9 ± 0.0	4.9 ± 0.1	2.1 ± 0.1	5.6 ± 0.2	2.4 ± 0.1	4.9 ± 0.1	2.5 ± 0.2

Table A.5.3. Validation parameters of venlafaxine analysis. Method detection and quantification limits (MDL, MQL) for fish tissues and feed are expressed in µg kg^{-1}, dry weight, and for fish plasma and water in µg L^{-1}.

Matrix	MDL	MQL	Recovery (%)	Precision (%RSD)	
				Intra-day	Inter-day
Brain	0.15	0.49	165	9.1	11.6
Liver	0.23	0.78	36	2.3	12.1
Muscle	0.43	1.43	106	2.2	2.8
Plasma	0.04	0.12	66	5.1	11
Feed	0.3	1	41	0.99	0.92
Water	0.15	0.49	—	3.6	—

Table A.5.4. VFX concentration in plasma (ug L^{-1}) of contaminated fish after 28 days of exposure via water (VFX-water treatments) and via feed (VFX-feed treatments) and after 7 days of elimination. Different letters indicate significant differences between treatments, whereas different symbols (* or #) indicate significant differences between day 28 and 35 for the same treatment ($p < 0.05$). Abbreviations: Acid – simulated acidification (i.e. pCO_2 ~1500 µatm, equivalent to pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC).

	Day 28	Day 35
VFX-water	$1291.6 \pm 79.9^{a*}$	$673.0 \pm 194.3^{b\#}$
Acid+Warm+VFX-water	1058.0 ± 64.8^{b}	1029 ± 90.7^{a}
VFX-feed	13.5 ± 1.4^{d}	9.6 ± 3.9^{d}
Acid+VFX-feed	24.8 ± 8.5^{cd}	30.0 ± 7.7^{c}
Warm+VFX-feed	34.9 ± 20.6^{cd}	23.3 ± 7.4^{cd}
Acid+Warm+VFX-feed	$40.6 \pm 11.7^{c*}$	$15.5 \pm 3.0^{d\#}$

ANNEX 6.

SUPPLEMENTARY INFORMATION FOR CHAPTER 4. PART 2.

Living in a multi-stressors environment: An integrated biomarker approach to assess the ecotoxicological response of meagre (Argyrosomus regius) to venlafaxine, warming and acidification

TABLES

Table A.6.1. Seawater physical and chemical parameters (mean ± standard deviation) in each treatment. Abbreviations: CTR – reference temperature and pH conditions (i.e. T = 19 ºC and pH = 8.0 units); VFX-feed – fish exposure VFX via feed; VFX-water –VFX exposure via water; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC); TA – total alkalinity; P CO_2 - partial CO_2 pressure; T CO_2 - Total CO_2 concentration; HCO_3^- - bicarbonate; CO_3^{2-} - carbonate ion concentrations; ΩCal - calcite saturation state; ΩAra – aragonite saturation state.

	Control	VFX-water	VFX-feed	Acid	Acid+VFX-feed	Warm	Warm+ VFX-feed	Acid+Warm	Acid+Warm+ VFX-feed	Acid+Warm+ VFX-water
Temperature (°C)	19.0 ± 0.2	19.0 ± 0.3	19.0 ± 0.1	19.0 ± 0.1	19.0 ± 0.1	24.0 ± 0.3	24.0 ± 0.2	24.0 ± 0.2	24.0 ± 0.2	24.0 ± 0.2
Salinity	35.2 ± 0.5	35.1 ± 0.7	35.1 ± 0.5	35.3 ± 0.6	35.1 ± 0.7	35.4 ± 0.8	35.5 ± 0.6	35.4 ± 0.8	35.3 ± 0.6	35.4 ± 0.8
pH	8.02 ± 0.01	8.05 ± 0.03	8.01 ± 0.04	7.61 ± 0.03	7.63 ± 0.02	8.01 ± 0.02	8.04 ± 0.03	7.62 ± 0.03	7.62 ± 0.04	7.62 ± 0.03
TA (µmol kg^{-1})	2737.1 ± 20.9	2705.9 ± 33.2	2715.4 ± 33.1	2687.5 ± 60.1	2648.0 ± 135.8	2624.0 ± 43.8	2681.2 ± 68.2	2565.5 ± 191.6	2494.5 ± 71.4	2565.5 ± 191.6
***P* CO_2** (µatm)	540.8 ± 4.6	534.6 ± 8.5	535.5 ± 6.8	1497 ± 32.9	1477.7 ± 78.4	509.5 ± 9.4	523.2 ± 12.7	1433.9 ± 107.8	1394.8 ± 39.0	1433.9 ± 107.8
***T* CO_2** (µmol kg^{-1})	2467.9 ± 21.2	2437.4 ± 33.8	2444.9 ± 29.3	2610.9 ± 59.7	2572.1 ± 134.5	2313.8 ± 41.9	2371.5 ± 60.1	2461.4 ± 185.9	2392.9 ± 68.2	2461.4 ± 185.9
HCO_3^- (µmol kg^{-1})	2242.7 ± 20.5	2214.0 ± 32.4	2220.1 ± 25.5	2470.8 ± 56.7	2434.0 ± 127.6	2066.7 ± 38.8	2121.9 ± 52.3	2316.5 ± 174.5	2252.4 ± 63.6	2316.5 ± 174.5
CO_3^{2-} (µmol kg^{-1})	207.3 ± 0.5	205.9 ± 1.0	207.3 ± 3.7	90.7 ± 1.7	89.4 ± 4.3	232.5 ± 2.8	234.4 ± 7.5	103.7 ± 8.4	100.4 ± 3.7	103.7 ± 8.4
Ω Ara	3.2 ± 0.1	3.2 ± 0.2	3.2 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	3.7 ± 0.0	3.7 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1
Ω Cal	4.9 ± 0.0	4.9 ± 0.1	4.9 ± 0.1	2.2 ± 0.0	2.1 ± 0.1	5.6 ± 0.1	5.6 ± 0.2	2.5 ± 0.2	2.4 ± 0.1	2.5 ± 0.2

Table A.6.2. Proximate chemical composition of CTR and VFX-enriched feeds (extracted from Maulvault et al., 2018b).

	%
Fishmeal LT70[1]	28.0
Fishmeal 60[2]	20.0
Fish protein concentrate[3]	2.5
Soy protein concentrate[4]	5.0
Wheat gluten[5]	5.5
Corn gluten[6]	5.0
Soybean meal 48[7]	9.0
Wheat meal[8]	5.0
Whole peas[9]	5.0
Fish oil[10]	13.5
Guar gum[11]	0.5
Vitamin and mineral premix[12]	1.0

[1] Peruvian fishmeal LT: 67% crude protein (CP), 9% crude fat (CF), EXALMAR, Peru.
[2] Fair Average Quality (FAQ) fishmeal: 62% CP, 12% CF, COFACO, Portugal.
[3] CPSP 90: 84% CP, 12% CF, Sopropêche, France.
[4] Soycomil P: 65% CP, 0.8% CF, ADM, The Netherlands.
[5] VITAL: 85.7% CP, 1.3% CF, ROQUETTE, France.
[6] GLUTALYS: 61% CP, 6 % CF, ROQUETTE, France.
[7] Dehulled solvent extracted soybean meal: 47% CP, 2.6% CF, Cargill, Spain.
[8] Whole wheat: 10.2% CP, 1.2% CF, Casa Lanchinha, Portugal.
[9] Whole peas: 19.8% CP, 2.2% CF, Casa Lanchinha, Portugal.
[10] SAVINOR UTS, Portugal
[11] Guar gum HV109, SEAH International, France.
[12] PREMIX Lda, Portugal: Vitamins: DL-alpha tocopherol acetate, 100 mg kg^{-1}; sodium menadione bisulphate, 25 mg kg^{-1}; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg kg^{-1}; riboflavin, 30 mg kg^{-1}; pyridoxine, 20 mg kg^{-1}; cyanocobalamin, 0.1 mg kg^{-1}; nicotinic acid, 200 mg kg^{-1}; folic acid, 15 mg kg^{-1}; ascorbic acid, 500 mg kg^{-1}; inositol, 500 mg kg^{-1}; biotin, 3 mg kg^{-1}; calcium panthotenate, 100 mg kg^{-1}; choline chloride, 1000 mg kg^{-1}, betaine, 500 mg kg^{-1}. Minerals: copper sulphate, 9 mg kg^{-1}; ferric sulphate, 6 mg kg^{-1}; potassium iodide, 0.5 mg kg^{-1}; manganese oxide, 9.6 mg kg^{-1}; sodium selenite, 0.01 mg kg^{-1}; zinc sulphate,7.5 mg kg^{-1}; sodium chloride, 400 mg kg^{-1}; excipient wheat middlings.

Table A.6.3. Tissue molecular biomarker levels in *A. regius* from CTR treatment (average ± standard deviation; n = 6). Abbreviations: CAT – catalase; SOD – superoxide dismutase; GST – glutathione S-transferase; LPO - lipid peroxidation; HSP70/HSC70 – heat shock proteins; Ub – total ubiquitin; AChE – acetylcholinesterase; VTG – vitellogenin content.

	CAT activity (U mg^{-1} protein)	**SOD activity (% inhibition)**	**GST activity (U mg^{-1} protein)**	**LPO (U mg^{-1} protein)**	**HSP70/HSC70 concentration (U mg^{-1} protein)**	**Ub concentration (U mg^{-1} protein)**	**VTG concentration (U mg^{-1} protein)**	**AChE activity (U mg^{-1} protein)**
Muscle	14.3 ± 1.2	80.9 ± 2.3	26.1 ± 3.4	0.005 ± 0.001	1.7 ± 0.1	0.14 ± 0.02	-	-
Gills	13.3 ± 4.7	86.7 ± 2.4	13.7 ± 3.3	0.014 ± 0.005	1.1 ± 0.1	0.08 ± 0.03	-	-
Liver	21.8 ± 2.3	77.2 ± 7.6	26.4 ± 3.7	0.016 ± 0.002	1.8 ± 0.2	0.07 ± 0.04	25.9 ± 3.1	-
Brain	4.5 ± 1.3	67.7 ± 5.3	9.7 ± 0.6	0.002 ± 0.001	0.6 ± 0.2	0.03 ± 0.01	-	419.0 ± 43.4

Table A.6.4. Molecular biomarker scores calculated in each treatment and fish tissue. Values in bold correspond to individual biomarker scores (S) that differed ≥ 0.5 from the CTR treatment score. Abbreviations: CAT – catalase activity; SOD – superoxide dismutase activity; GST – glutathione S-transferase activity; LPO - lipid peroxidation, measured as MDA concentration; HSP70/HSC70 - heat shock proteins concentration; Ub - total ubiquitin concentration; VTG – vitellogenin concentration; AChE – acetylcholinesterase activity; CTR – reference temperature and pH conditions (i.e. T = 19 ºC and pH = 8.0 units); VFX-feed – VFX exposure via feed; VFX-water – VFX exposure via water; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC).

		CAT	SOD	GST	LPO	HSP70/HSC70	Ub	VTG	AChE
CTR	**Muscle**	2.79	2.34	0.62	0.52	1.00	1.31	-	-
	Gills	2.17	1.31	2.80	0.31	0.93	0.63	-	-
	Liver	0.49	1.56	2.02	1.71	1.15	0.91	1.42	-
	Brain	0.74	1.29	0.21	1.16	0.14	0.46	-	0.19
VFX-Feed	**Muscle**	**1.30**	**4.95**	**2.17**	**2.32**	**1.94**	**2.94**	**-**	**-**
	Gills	**3.81**	**4.67**	**2.24**	**2.98**	**1.56**	**1.71**	**-**	**-**
	Liver	**3.60**	**2.98**	2.30	**2.92**	1.48	**3.02**	**3.42**	**-**
	Brain	**2.02**	**0.52**	**1.85**	**3.35**	**1.71**	**2.26**	**-**	**2.16**
VFX-water	**Muscle**	**0.74**	**4.49**	**3.21**	**1.45**	**2.41**	**2.76**	**-**	**-**
	Gills	**2.69**	**4.45**	**2.08**	**2.39**	**3.09**	**2.47**	**-**	**-**
	Liver	**1.21**	**3.49**	**2.71**	**3.78**	**1.72**	**3.79**	**2.17**	**-**
	Brain	0.68	**2.72**	**2.33**	**3.13**	**3.09**	**4.28**	**-**	**2.57**
Acid	**Muscle**	**1.60**	**3.98**	0.20	**1.87**	0.65	**2.17**	-	-
	Gills	**1.32**	**4.14**	**4.67**	**2.28**	0.50	**3.49**	-	-
	Liver	0.56	**2.80**	**2.81**	**2.44**	**3.34**	**2.96**	**3.16**	**-**
	Brain	0.41	**2.62**	**2.31**	**4.05**	**1.95**	**2.05**	**-**	**2.12**
Acid+VFX-feed	**Muscle**	**0.79**	**4.53**	**2.14**	**3.20**	**1.92**	**2.77**	**-**	**-**
	Gills	**1.06**	**4.08**	**1.89**	**2.23**	**1.59**	**1.55**	**-**	**-**
	Liver	**1.16**	**4.35**	2.04	**3.85**	**1.80**	**3.52**	**2.10**	**-**
	Brain	**1.78**	**2.80**	**1.96**	**3.24**	**1.98**	**2.03**	**-**	**1.58**
Warm	**Muscle**	**1.61**	**5.22**	**2.18**	0.29	**2.60**	**3.71**	-	-
	Gills	**2.28**	**4.26**	2.31	**2.32**	0.78	**1.60**	-	-
	Liver	**1.34**	**2.81**	1.77	**3.93**	**2.42**	**2.32**	**2.18**	**-**
	Brain	**2.38**	**3.38**	**3.39**	**4.33**	**2.26**	**1.93**	**-**	**2.80**
Warm+VFX-feed	**Muscle**	**1.18**	**3.64**	**2.54**	**1.80**	**3.25**	**2.19**	**-**	**-**
	Gills	**2.73**	**4.91**	**0.61**	**2.39**	**1.49**	0.30	-	-
	Liver	**1.55**	**3.51**	**2.92**	**2.70**	**2.55**	**3.00**	**3.41**	**-**
	Brain	**2.29**	**2.15**	**1.64**	**2.86**	**1.82**	**1.77**	**-**	**1.56**
Acid+Warm	**Muscle**	**1.02**	**4.82**	**2.19**	**1.58**	0.76	**2.37**	-	-
	Gills	**1.97**	**4.39**	2.37	**2.49**	**0.24**	**1.55**	**-**	**-**
	Liver	**1.13**	**3.56**	**2.63**	**2.49**	**2.26**	**2.45**	**1.95**	**-**
	Brain	**1.89**	**3.00**	**1.42**	**2.83**	**2.57**	**2.07**	**-**	**2.87**
Acid+Warm+VFX-feed	**Muscle**	**3.49**	**3.77**	**2.72**	**2.92**	0.69	**2.62**	-	-
	Gills	**2.86**	**4.08**	2.34	**3.49**	**1.47**	**0.38**	**-**	**-**
	Liver	**1.72**	**3.78**	1.99	**3.03**	**2.89**	**2.72**	1.85	-
	Brain	**2.14**	**2.64**	**1.56**	**2.91**	**3.46**	**1.99**	**-**	**1.69**
Acid+Warm+VFX-water	**Muscle**	**0.25**	**4.02**	**3.28**	**2.08**	**3.06**	**3.90**	**-**	**-**
	Gills	**3.95**	**4.29**	2.35	**2.69**	**3.13**	**1.95**	**-**	**-**
	Liver	**1.64**	**4.06**	**2.73**	**2.93**	**2.17**	**2.95**	**3.10**	**-**
	Brain	**3.78**	**3.40**	**2.05**	**4.01**	**2.06**	**2.31**	**-**	**2.66**

METHODOLOGIES

1. Total protein content

Bradford assay (Bradford, 1976) was carried out to quantify total protein levels in each sample, and to enable the subsequent normalization of biomarker (i.e. given in mg of protein). In this assay, 180 µL of Bradford reagent (Bradford, 1976) and 20 µL of sample/standard were added to each microplate well. A calibration curve was generated with bovine serum albumin (BSA; Sigma Aldrich, Germany) at different dilutions (at least 7 concentrations, ranging from 0 to 2.0 mg mL^{-1}) as standard. Absorbance was read at 595 nm in a microplate reader (BioRad, Benchmark, USA).

2. Catalase (CAT)

Catalase activity (EC 1.11.1.6) was carried out following the procedure described by Johansson and Borg (1988), and adapted to 96-well microplates. To each microplate well, 20 µL of sample/standard, 100 µL of assay buffer (100 mM potassium phosphate; Sigma Aldrich, Germany), 30 µL of methanol (Merck, USA) and 20 µL of hydrogen peroxide (0.035 M; Sigma-Aldrich, Germany) were added. After 20 min incubation, 30 µL of potassium hydroxide (10 M; Merck, USA) and 30 µL of Purpald (34.2 mM; Merck, USA) were added. After incubating 10 min, 10 µL of potassium periodate (65.2 mM; Merck, USA) were added to finalize the reaction. A calibration curve was built using formaldehyde standards, with concentrations ranging from 5 to 75 µM of formaldehyde (Sigma Aldrich, Germany). Standard bovine catalase solution of 1523.6 U mL^{-1} (Sigma Aldrich, Germany) was used as positive control. Enzyme activity was calculated considering that one unit of catalase is defined as the amount that will cause the formation of 1.0 nmol of formaldehyde per minute at 25 °C. Absorbance was read at 540 nm and results expressed as µM min^{-1} mg $protein^{-1}$.

3. Superoxide dismutase (SOD)

Superoxide dismutase activity (EC 1.15.1.1) was carried out as described by Sun et al. (1988), and adapted to 96-well microplates. Briefly, 10 µL of sample were transferred to the microplate wells. In each well, 240 µL of a reagent mix (3 mM EDTA, Merck, USA; 3 mM xanthine, Sigma Aldrich, Germany; 0.75 mM NBT, Merck, USA; and 100 um XOD Sigma Aldrich, Germany) were added. Negative controls (i.e. mix without sample) were also included. Samples absorbance was read at 550 nm, and results were presented as the percentage of enzyme inhibition, using the following equation:

$$\%\ inhibition = \frac{Abs550/min_{negative\ control} - Abs550/min_{sample}}{Abs550/min_{negative\ control}}\ x\ 100$$

4. Glutathione S-transferase (GST)

Glutathione S-transferase activity (EC 2.5.1.18) was determined according to the method described by Habig et al. (1974) and adapted to 96-well microplates. To each microplate well, 20 µL of sample/standard and 180 µL of a reagent mix (200 mM reduced L-glutathione, 100 mM CNDB and buffer Dulbecco; all from Sigma-Aldrich, Germany) were added. Equine liver GST (Sigma-Aldrich, Germany) was used as standard and positive control. Absorbance was read at 340 nm every minute during 6 minutes, with the increase in absorbance being directly proportional to GST activity. The reaction rate was determined considering the molar CDNB extinction coefficient of $0.0053\varepsilon^{mM}$, and results were expressed as nmol min^{-1} mg $protein^{-1}$.

5. Lipid peroxidation (LPO)

Lipid peroxidation was determined using a 96-well microplates protocol adapted from the thiobarbituric acid reactive substances (TBARS) method of Uchiyama and Mihara (1978). 5 µL of each sample/standard were added to microtubes. Then, 45 µL of monobasic sodium phosphate buffer (50 mM, Sigma Aldrich, Germany), 12.5 µL of SDS (8.1%, Sigma Aldrich, Germany), 93.5 µL of trichloroacetic acid (20%, pH1/43.5, Merck, USA), 93.5 µL of thiobarbituric acid (1%, Merck USA) and 50.5 µL of Milli-Q grade ultrapure water were added to each microtube. Microtubes were then vortexed for 30 s and incubated in boiling water for 10 min (microtubes' lids were punctured with a needle before incubation). Afterwards, microtubes were immediately placed on ice for a few minutes to cool to room temperature, and 62.5 µL of Milli-Q grade ultrapure water and 312.5 µL of n-butanol pyridine (15:1, v/v, MercK, USA) were added. Microtubes were vortexed again, and centrifuged at 10,000 *g* for 5 min. Duplicates of 150 µL of the supernatant of each reaction were transferred to microplate wells. Lipid peroxides were quantified using an eight-point calibration curve ranging from 0 to 0.3 mM TBARS, performed with malondialdehyde bis (dimethylacetal) standards (Merck). Absorbance was read at 530 nm and results were expressed as nmol mg^{-1} protein.

6. Heat shock response (HSP70/HSC70)

Heat Shock Protein 70 (HSP70/HSC70) was quantified using an indirect Enzyme Linked Immunosorbent Assay (ELISA) based on a protocol from Njemini et al. (2005). Briefly, 50 µL of sample/standard were added to microplate wells, and left to incubate overnight at 4°C. Afterwards, microplates were washed 3 times with a PBS solution containing 0.05% of Tween-20 (Sigma-Aldrich, Germany), and then blocked by adding 200 µL of 1% BSA solution prepared in PBS. Microplates were incubated for 90 min at 37 ºC and washed again (with PBS+0.05% of Tween-20 solution) and 50 µL of primary antibody solution were added to each well (anti-Hsp70/Hsc70, Acris, USA; diluted to 1.0 µg mL^{-1} in a 1% BSA solution). Then, microplates were incubated again for 90 min at 37 ºC, washed (with PBS+0.05% of Tween-20 solution) and 50 µL

of secondary antibody solution were added to each well (anti-mouse IgG, fab specific, alkaline phosphatase conjugate, Sigma-Aldrich, Germany; also diluted to 1.0 µg mL^{-1} in 1% BSA solution). After incubating at 37 °C for 90 min, microplates were washed again, 100 µL of substrate (SIGMA FAST™ p-Nitrophenyl Phosphate Tablets, Sigma- Aldrich, USA) was added and microplates were left to incubate for 30 min at room temperature. Then, 50 µL of NaOH solution (3N, Merck, Germany) were added to stop the reaction.

A calibration curve was performed using serial dilutions (at least 7 different concentrations) of purified HSP70 active protein (Acris, USA), ranging from 0 to 2.0 µg mL^{-1} of protein. The absorbance was read at 405 nm and results were expressed in µg mg^{-1} protein.

7. Ubiquitin (Ub)

Total ubiquitin was quantified using a direct ELISA method previously described by Madeira et al. (2014). Briefly, 50 µL of sample/standard were added to microplate wells, and left to incubate overnight at 4°C. Afterwards, microplates were washed 3 times with a PBS solution containing 0.05% of Tween-20, and then blocked by adding 200 µL of 1% BSA solution prepared in PBS. Microplates were incubated for 90 min at 37 ºC and washed again (with PBS+0.05% of Tween-20 solution) and 50 µL of a conjugated primary antibody (Ub P4D1, sc-8017, HRP conjugate, Santa Cruz, USA) were added to each well. Microplates were incubated for 90 min at 37 ºC, washed again (with PBS+0.05% of Tween-20 solution), and 100 µL of substrate (TMB/E, Temecula California, Merck Millipore) was added. After incubating for 30 min at room temperature, 100 µL stop solution (1 N HCl, Merck, USA) was added. A calibration curve was performed using serial dilutions (at least 7 concentrations) of the ubiquitin standard E110, UbP (Santa Cruz, USA; concentrations ranging from 0 to 2.0 µg mL^{-1} protein). The absorbance was read at 450 nm and results were expressed in µg mg^{-1} protein.

8. Acetylcholinesterase (AChE) activity

Acetylcholinesterase (EC 3.1.1.7) activity was assessed in brain tissues, following a methodology adapted from Ellman et al. (1961) to 96-well microplates. Briefly, 50 µL of previously diluted samples (1:10) were transferred to the microplate wells, and 250 µL of a reagent mix containing (sodium phosphate buffer 50 mM at 8.0 pH units, 1 mM Ellman's reagent, i.e. DTNB – 5,5'-dithiobis-(2-nitrobenzoic acid; in 50mM phosphate buffer, and 75 mM ACTI (acetilthiocholine iodide); in phosphate buffer 50 mM; all from Sigma-Aldrich, Germany) were added. Negative controls were included (i.e. 50 µL of buffer instead of sample). Samples absorbance was read at 412 nm, every minute during 10 minutes, and AChE activity was measured considering that one unit of enzyme is responsible for the formation of 1.0 µmol of thiocholine per minute. Results were expressed as nmol min^{-1} mg $protein^{-1}$.

9. Vitellogenin (VTG)

Vitellogenin content (VTG) was determined in fish liver following a direct ELISA assay based on the protocol of Denslow et al. (1999). Briefly, 50 µL of sample (previously diluted 1:200) or standard were added to microplate wells, and left to incubate overnight at 4°C. Afterwards, microplates were washed 3 times with a TBST solution (10 mM Tris-HCl at 7.0 pH units, Merck, USA; 150 mM NaCl, Merck, USA; and 0,05% de Tween-20, Sigma-Aldrich, Germany), and then blocked by adding 200 µL of 1% BSA solution prepared in PBS. Microplates were incubated for 90 min at 37 ºC and washed again (with TBST solution) and 50 µL of a primary antibody (carp VTG monoclonal antibody, Biosense, Norway; diluted to 1.0 µg mL^{-1} in a 1% BSA solution) were added. Then, microplates were incubated again for 90 min at 37 ºC, washed (TBST solution) and 50 µL of secondary antibody solution (anti-mouse IgG, fab specific, alkaline phosphatase conjugate, Sigma-Aldrich, Germany; also diluted to 1.0 µg mL^{-1} in 1% BSA) were added to each well. After incubating at 37 °C for 90 min, microplates were washed again, 100 µL of substrate (SIGMA FAST™ p-Nitrophenyl Phosphate Tablets, Sigma- Aldrich, USA) was added and microplates were left to incubate for 30 min at room temperature. Then, 50 µL of NaOH solution (3N) were added to stop the reaction. A calibration curve was performed using serial dilutions (at least 7 concentrations) of carp VTG standard (Biosense, Norway), ranging from 0 to 2.0 µg mL^{-1} of protein. The absorbance was read at 405 nm and results were expressed in µg mg^{-1} protein.

10. Integrated Biomarker Response (IBR)

Integrated biomarker responses (IBRs) and respective star plots were calculated in order to understand the global ecotoxicological responses of *A. regius* exposed to VFX (via feed and water), acidification and warming. Calculations were performed according to the methodology proposed by Beliaeff and Burgeot (2002), later modified by Guerlet et al. (2010). Briefly, for each tissue, the general mean (*m*) and standard deviation (*s*) of a given biomarker was calculated (including data from all treatments), and subsequently standardized to obtain Y, i.e. Y = (X – *m*) / *s*, where X is the mean biomarker value of a given treatment. Then, Z was calculated as Z = -Y or Z = +Y according to the expected biological effect, with "-" representing an inhibition of a biological effect and "+" representing an induction (such evaluation was based on the average baseline biomarker values). Then, biomarker scores (S) were calculated as S = Z + |Min|, where Z ≥ 0 and |Min| is the absolute value of all Y calculated for a given biomarker (including all measurements). Star plots were performed to represent the scores (S) of all biomarkers measured in a given treatment and tissue, as well as to calculate IBRs according to the following formulas:

$$IBR = \sum_{t=1}^{n} A_i$$

$$A_i = \frac{1}{2}\sin\left(\frac{2\,x\,\pi}{n}\right) x\, S_i\, x\, S_{i+1}$$

where, A_i is the area connecting the two scores (S), S_i and S_{i+1} are two consecutive clockwise scores (radius coordinates) of a given star plot, and *n* is the number of biomarkers used for calculations. IBR calculations were always performed with the same order of parameters for all treatments and tissues, i.e. CAT, SOD, GST, LPO, HSP70/HSC70, Ub, and VTG (in the case of fish liver) or AChE (in the case of fish brain). In order to evaluate the scores as a fitness index, values that differed in 0.5 from the score of CTR treatment were considered to be from an animal with a higher or lower fitness (Ferreira et al., 2015).

ANNEX 7.

SUPPLEMENTARY INFORMATION FOR CHAPTER 4. PART 3.

Differential behavioural responses to venlafaxine exposure route, warming and acidification in juvenile fish (Argyrosomus regius)

FIGURES

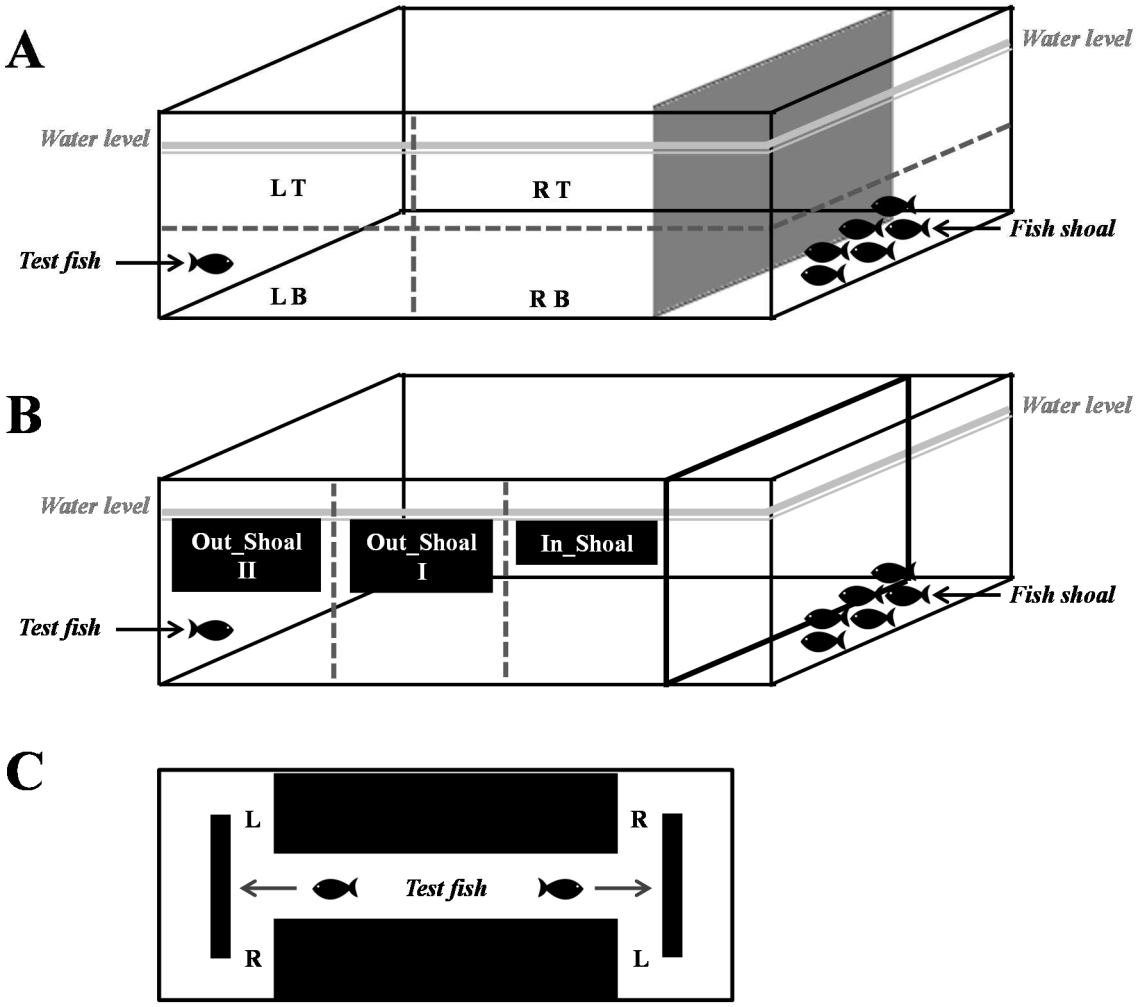

Figure A.7.1. Tank setup for behavior tests: 1 - Novel tank assay (A), 2 – Shoaling Assay (B) and 3 – Lateralization (C). Abbreviations: LB – Left-bottom area; LT – Left-top area of the tank; RB – Right-bottom area of the tank; RT – Right-top area of the tank; in_shoal – Tank area closer to the shoal (i.e. test fish considered to be inside the shoal); out_shoal-I – Tank area far from shoal (i.e. test fish considered to be outside the shoal); out_shoal-II – Tank area very far from shoal (i.e. test fish considered to be outside the shoal); L – Left side (fish perspective); R – Right side (fish perspective).

TABLES

Table A.7.1. Proximate chemical composition of CRT and VFX-enriched feeds used in trials I and II.

	%
Fishmeal LT70[1]	28.0
Fishmeal 60[2]	20.0
Fish protein concentrate[3]	2.5
Soy protein concentrate[4]	5.0
Wheat gluten[5]	5.5
Corn gluten[6]	5.0
Soybean meal 48[7]	9.0
Wheat meal[8]	5.0
Whole peas[9]	5.0
Fish oil[10]	13.5
Guar gum[11]	0.5
Vitamin and mineral premix[12]	1.0

[1] Peruvian fishmeal LT: 67% crude protein (CP), 9% crude fat (CF), EXALMAR, Peru.
[2] Fair Average Quality (FAQ) fishmeal: 62% CP, 12% CF, COFACO, Portugal.
[3] CPSP 90: 84% CP, 12% CF, Sopropêche, France.
[4] Soycomil P: 65% CP, 0.8% CF, ADM, The Netherlands.
[5] VITAL: 85.7% CP, 1.3% CF, ROQUETTE, France.
[6] GLUTALYS: 61% CP, 6 % CF, ROQUETTE, France.
[7] Dehulled solvent extracted soybean meal: 47% CP, 2.6% CF, Cargill, Spain.
[8] Whole wheat: 10.2% CP, 1.2% CF, Casa Lanchinha, Portugal.
[9] Whole peas: 19.8% CP, 2.2% CF, Casa Lanchinha, Portugal.
[10] SAVINOR UTS, Portugal
[11] Guar gum HV109, SEAH International, France.
[12] PREMIX Lda, Portugal: Vitamins: DL-alpha tocopherol acetate, 100 mg kg^{-1}; sodium menadione bisulphate, 25 mg kg^{-1}; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg kg^{-1}; riboflavin, 30 mg kg^{-1}; pyridoxine, 20 mg kg^{-1}; cyanocobalamin, 0.1 mg kg^{-1}; nicotinic acid, 200 mg kg^{-1}; folic acid, 15 mg kg^{-1}; ascorbic acid, 500 mg kg^{-1}; inositol, 500 mg kg^{-1}; biotin, 3 mg kg^{-1}; calcium panthotenate, 100 mg kg^{-1}; choline chloride, 1000 mg kg^{-1}, betaine, 500 mg kg^{-1}. Minerals: copper sulphate, 9 mg kg^{-1}; ferric sulphate, 6 mg kg^{-1}; potassium iodide, 0.5 mg kg^{-1}; manganese oxide, 9.6 mg kg^{-1}; sodium selenite, 0.01 mg kg^{-1}; zinc sulphate,7.5 mg kg^{-1}; sodium chloride, 400 mg kg^{-1}; excipient wheat middlings.

Table A.7.2. Seawater physical and chemical parameters (mean ± standard deviation) in each treatment of Trials I and II. Abbreviations: Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC); VFX-water – fish exposed to VFX via water; VFX-feed – fish exposed to VFX via feed; TA – total alkalinity; pCO_2 - partial CO_2 pressure; TCO_2 - Total CO_2 concentration; HCO_3^- - bicarbonate; CO_3^{2-} - carbonate ion concentrations; ΩCal - calcite saturation state; ΩAra – aragonite saturation state.

Trial I	**Control**	**VFX-water**	**VFX-feed**				
Temperature (°C)	19.0 ± 0.2	19.0 ± 0.3	19.0 ± 0.1				
Salinity	35.2 ± 0.5	35.1 ± 0.7	35.1 ± 0.5				
pH	8.02 ± 0.01	8.05 ± 0.03	8.01 ± 0.04				
TA (µmol kg^{-1})	2737.1 ± 20.9	2705.9 ± 33.2	2715.4 ± 33.1				
***p*CO$_2$ (µatm)**	540.8 ± 4.6	534.6 ± 8.5	535.5 ± 6.8				
***T*CO$_2$ (µmol kg^{-1})**	2467.9 ± 21.2	2437.4 ± 33.8	2444.9 ± 29.3				
***HCO$_3^-$* (µmol kg^{-1})**	2242.7 ± 20.5	2214.0 ± 32.4	2220.1 ± 25.5				
CO$_3^{2-}$ (µmol kg^{-1})	207.3 ± 0.5	205.9 ± 1.0	207.3 ± 3.7				
Ω Ara	3.2 ± 0.1	3.2 ± 0.2	3.2 ± 0.1				
Ω Cal	4.9 ± 0.0	4.9 ± 0.1	4.9 ± 0.1				
Trial II	**VFX-Feed**	**Acid**	**Acid+VFX-feed**	**Warm**	**Warm+VFX-feed**	**Acid+Warm**	**Acid+Warm+VFX-feed**
Temperature (°C)	19.0 ± 0.2	19.0 ± 0.1	19.0 ± 0.1	24.0 ± 0.3	24.0 ± 0.2	24.0 ± 0.2	24.0 ± 0.2
Salinity	35.1 ± 0.7	35.3 ± 0.6	35.1 ± 0.7	35.4 ± 0.8	35.5 ± 0.6	35.4 ± 0.8	35.3 ± 0.6
pH	8.02 ± 0.02	7.61 ± 0.03	7.63 ± 0.02	8.01 ± 0.02	8.04 ± 0.03	7.62 ± 0.03	7.62 ± 0.04
TA (µmol kg^{-1})	2715.4 ± 33.1	2687.5 ± 60.1	2648.0 ± 135.8	2624.0 ± 43.8	2681.2 ± 68.2	2565.5 ± 191.6	2494.5 ± 71.4
***p*CO$_2$ (µatm)**	535.5 ± 6.8	1497 ± 32.9	1477.7 ± 78.4	509.5 ± 9.4	523.2 ± 12.7	1433.9 ± 107.8	1394.8 ± 39.0
***T*CO$_2$ (µmol kg^{-1})**	2444.9 ± 29.3	2610.9 ± 59.7	2572.1 ± 134.5	2313.8 ± 41.9	2371.5 ± 60.1	2461.4 ± 185.9	2392.9 ± 68.2
***HCO$_3^-$* (µmol kg^{-1})**	2220.1 ± 25.5	2470.8 ± 56.7	2434 ± 127.6	2066.7 ± 38.8	2121.9 ± 52.3	2316.5 ± 174.5	2252.4 ± 63.6
CO$_3^{2-}$ (µmol kg^{-1})	207.3 ± 3.7	90.7 ± 1.7	89.4 ± 4.3	232.5 ± 2.8	234.4 ± 7.5	103.7 ± 8.4	100.4 ± 3.7
Ω Ara	3.2 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	3.7 ± 0.0	3.7 ± 0.1	1.6 ± 0.1	1.6 ± 0.1
Ω Cal	4.9 ± 0.1	2.2 ± 0.0	2.1 ± 0.1	5.6 ± 0.1	5.6 ± 0.2	2.5 ± 0.2	2.4 ± 0.1

Table A.7.3. Validation parameters of venlafaxine analysis in feed, water and fish plasma. Method detection and quantification limits (MDL, MQL) for feed are expressed in µg kg^{-1}, dry weight, and for water and fish plasma in µg L^{-1}.

Matrix	MDL	MQL	Recovery (%)	Precision (%RSD)	
				Intra-day	Inter-day
Feed	0.30	1.0	41	0.99	0.92
Water	0.15	0.49	—	3.6	—
Fish plasma	0.04	0.12	66	5.1	11.0

Table A.7.4. General linear mixed models (GLMMs), with tank as random effect, of behavior results in juvenile *A. regius*. Values in bold indicate significant differences. Abbreviations: Est – estimates; Std error – standard error.

Trial I	**GLMM - Latency to top**				**GLMM - Latency to shoal**				**GLMM - L_A**				**GLMM - L_R**			
	Est	**Std error**	***t* value**	***p* value**	**Est**	**Std error**	***t* value**	***p* value**	**Est**	**Std error**	***t* value**	***p* value**	**Est**	**Std error**	***t* value**	***p* value**
Intercept	119.00	10.42	11.42	**< 0.001**	2.20	0.56	3.96	**< 0.001**	34.00	6.12	5.55	**< 0.001**	-34.00	6.12	5.55	**< 0.001**
VFX (Control)	-42.90	14.74	-2.91	**< 0.01**	3.60	0.79	4.59	**< 0.001**	30.00	8.66	3.47	**< 0.01**	-30.00	8.66	3.47	**< 0.01**
VFX (Water)	-86.10	14.74	-5.84	**< 0.001**	2.80	0.79	3.57	**< 0.01**	22.00	8.66	2.54	**< 0.05**	-22.00	8.66	2.54	**< 0.05**
	Familiy = Gaussian				Familiy = Gaussian				Familiy = Gaussian				Familiy = Gaussian			
Trial I	**GLMM - % time spent in top**				**GLMM - Total transitions (activity)**				**GLMM - % transitions to shoal**				**GLMM - % time spent within shoal**			
	Est	**Std error**	***z* value**	***p* value**	**Est**	**Std error**	***z* value**	***p* value**	**Est**	**Std error**	***z* value**	***p* value**	**Est**	**Std error**	***z* value**	***p* value**
Intercept	-2.17	0.13	-17.19	**< 0.001**	2.1401	0.2083	10.272	**< 0.001**	-0.03	0.13	-0.20	0.8420	2.51	0.09	27.18	**< 0.001**
VFX (Control)	1.62	0.17	9.43	**< 0.001**	1.0986	0.281	3.909	**< 0.001**	0.16	0.21	0.79	0.4290	-3.32	0.12	-28.21	**< 0.001**
VFX (Water)	3.56	0.17	20.49	**< 0.001**	0.1625	0.2916	0.557	0.577	0.05	0.18	0.27	0.7840	-1.96	0.12	-16.80	**< 0.001**
	Familiy = binomial				Familiy = negative binomial				Familiy = binomial				Familiy = binomial			
Trial II	**GLMM - Latency to top**				**GLMM - Latency to shoal**				**GLMM - L_A**				**GLMM - L_R**			
	Est	**Std error**	***t* value**	***p* value**	**Est**	**Std error**	***t* value**	***p* value**	**Est**	**Std error**	***t* value**	***p* value**	**Est**	**Std error**	***t* value**	***p* value**
Intercept	76.10	7.92	9.61	**< 0.001**	5.80	0.70	8.27	**< 0.001**	64.00	5.06	12.65	**< 0.001**	-63.95	8.17	-7.83	**< 0.001**
***p*CO₂**	12.50	11.20	1.12	0.2681	-0.80	0.99	-0.81	0.4223	-30.00	7.16	-4.19	**< 0.001**	98.42	11.55	8.52	**< 0.001**
Temp	-46.30	11.20	-4.13	**< 0.001**	-2.90	0.99	-2.93	**< 0.01**	-42.00	7.16	-5.87	**< 0.001**	57.43	11.28	5.09	**< 0.001**
VFX	42.90	11.20	3.83	**< 0.001**	-3.60	0.99	-3.63	**< 0.001**	-30.00	7.16	-4.19	**< 0.001**	30.00	11.55	2.60	**< 0.05**
***p*CO₂ xTemp**	-13.50	15.84	-0.85	0.3969	7.40	1.40	5.28	**< 0.001**	40.00	10.12	3.95	**< 0.001**	-108.11	15.85	-6.82	**< 0.001**
***p*CO₂ x VFX**	166.40	15.84	10.51	**< 0.001**	1.00	1.40	0.71	0.4780	16.00	10.12	1.58	0.1183	-44.47	16.33	-2.72	**< 0.05**
Temp x VFX	1.80	15.84	0.11	0.9098	3.10	1.40	2.21	**< 0.05**	58.00	10.12	5.73	**< 0.001**	-73.48	16.14	-4.55	**< 0.001**
***p*CO₂ xTempxVFX**	46.90	22.40	2.09	**< 0.05**	4.40	1.98	2.22	**< 0.05**	-66.00	14.31	-4.61	**< 0.001**	109.96	22.75	4.83	**< 0.001**
	Familiy = Gaussian				Familiy = Gaussian				Familiy = Gaussian				Familiy = Gaussian			
Trial II	**GLMM - % time spent in top**				**GLMM - Total transitions (activity)**				**GLMM - % transitions to shoal**				**GLMM - % time spent within shoal**			
	Est	**Std error**	***z* value**	***p* value**	**Est**	**Std error**	***z* value**	***p* value**	**Est**	**Std error**	***z* value**	***p* value**	**Est**	**Std error**	***z* value**	***p* value**
Intercept	-0.55	0.14	-4.04	**< 0.001**	3.24	0.15	22.01	**< 0.001**	0.14	0.16	0.87	0.3860	-0.81	0.13	-6.13	**< 0.001**
***p*CO₂**	-0.95	0.19	-4.86	**< 0.001**	-0.39	0.21	-1.85	0.0640	-0.05	0.22	-0.23	0.8180	0.98	0.19	5.30	**< 0.001**
Temp	-3.91	0.25	-15.79	**< 0.001**	1.05	0.20	5.18	**< 0.001**	-0.05	0.18	-0.28	0.7820	-0.80	0.17	-4.63	**< 0.001**
VFX	-1.63	0.20	-8.20	**< 0.001**	-1.10	0.23	-4.86	**< 0.001**	-0.16	0.21	-0.79	0.4290	3.33	0.19	17.09	**< 0.001**
***p*CO₂ x Temp**	-0.55	0.47	-1.17	0.2400	-0.21	0.29	-0.72	0.4730	-0.13	0.27	-0.48	0.6310	1.77	0.23	7.79	**< 0.001**
***p*CO₂ x VFX**	-3.10	0.49	-6.28	**< 0.001**	0.23	0.33	0.70	0.4850	-0.01	0.28	-0.05	0.9640	-2.12	0.27	-7.88	**< 0.001**
Temp x VFX	2.17	0.34	6.36	**< 0.001**	-2.35	0.36	-6.49	**< 0.001**	-0.22	0.26	-0.88	0.3800	-2.17	0.26	-8.34	**< 0.001**
***p*CO₂ xTempxVFX**	5.83	0.69	8.42	**< 0.001**	2.90	0.48	6.08	**< 0.001**	0.19	0.37	0.52	0.6050	0.96	0.35	2.73	**< 0.01**
	Familiy = binomial				Familiy = negative binomial				Familiy = binomial				Familiy = binomial			

ANNEX 8.

SUPPLEMENTARY INFORMATION FOR CHAPTER 5. PARTS 1. AND 2.

Bioaccumulation and ecotoxicological responses of juvenile white seabream (Diplodus sargus) exposed to triclosan, warming and acidification

TABLES

Table A.8.1. Proximate chemical composition of CRT and TCS-contaminated feeds.

Ingredients	%
Fishmeal LT70[1]	40.0
Fish protein concentrate[2]	7.8
Squid meal[3]	10.0
Chlorella[4]	2.0
Soy protein concentrate[5]	4.0
Soybean meal 48[6]	4.0
Wheat meal[7]	6.0
Fish oil[8]	12.0
Soy lecithin[9]	2.0
Guar gum[10]	1.2
Macroalgae[11]	5.0
Antioxidant[12]	0.5
Monocalcium phosphate[13]	1.0
Glycerol[14]	3.5
Vitamin and mineral premix[15]	1.0

[1] Peruvian fishmeal LT: 67% crude protein (CP), 9% crude fat (CF), EXALMAR, Peru;
[2] CPSP 90: 84% CP, 12% CF, Sopropêche, France;
[3] Super prime without guts: 82% CP, 3.5% CF, Sopropêche, France;
[4] Chlorella powder: 62.5% CP, 9.2% CF, ALLMICROALGAE, Portugal;
[5] Soycomil P: 65% CP, 0.8% CF, ADM, The Netherlands;
[6] Dehulled solvent extracted soybean meal: 47% CP, 2.6% CF, Cargill, Spain;
[7] Whole wheat: 10.2% CP, 1.2 % CF, Casa Lanchinha, Portugal;
[8] SAVINOR UTS, Portugal;
[9] LECICO P 700IPM, LECICO GmbH, Germany;
[10] Guar gum HV109, SEAH International, France;
[11] OceanFeed: 10.2% CP, Ocena Harvest Technology, Ireland;
[12] Paramega PX, Kemin Europe NV, Belgium;
[13] Monocalcium phosphate: 22% phosphorus, 16% calcium, Fosfitalia, Italy;
[14] Rapeseed-derived crude glycerol, IBEROL, Portugal;
[15] PREMIX Lda, Portugal: Vitamins (IU or mg kg^{-1} diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg; Minerals (g or mg kg^{-1} diet): copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; excipient wheat middlings.

Table A.8.2. Seawater physical and chemical parameters (mean ± standard deviation) in each treatment. Abbreviations: Control – reference temperature and pH conditions (i.e. T = 19 ºC and pH = 8.0 units); Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC); TCS – Triclosan exposure; TA – total alkalinity; pCO_2 - partial CO_2 pressure; TCO_2 - Total CO_2 concentration; HCO_3^- - bicarbonate; CO_3^{2-} - carbonate ion concentrations; ΩCal - calcite saturation state; ΩAra – aragonite saturation state.

	Control	Acid	Warm	Acid+Warm	TCS	TCS+Acid	TCS+Warm	TCS+Acid+Warm
Temperature (°C)	19.6 ± 0.5	19.4 ± 0.4	24.2 ± 0.2	24.2 ± 0.4	19.5 ± 0.5	19.1 ± 0.2	24.2 ± 0.2	23.9 ± 0.1
Salinity	35.4 ± 0.3	35.2 ± 0.4	35.2 ± 0.1	35.1 ± 0.1	35.1 ± 0.2	35.0 ± 0.1	35.0 ± 0.2	35.3 ± 0.3
pH	8.01 ± 0.12	7.63 ± 0.22	8.05 ± 0.10	7.60 ± 0.31	8.08 ± 0.09	7.59 ± 0.13	8.00 ± 0.10	7.61 ± 0.20
TA (µmol kg^{-1})	2818.9 ± 34.9	2780 ± 36.8	2752.2 ± 89.1	2653.4 ± 69.9	2618.9 ± 34.9	2753.4 ± 76.3	2652.2 ± 118.1	2786.7 ± 54.9
pCO_2 (µatm)	489.0 ± 53.7	1535.8 ± 90.9	496.5 ± 43.6	1522.8 ± 16.5	506.2 ± 134.9	1510.3 ± 139.2	524.9 ± 141.3	1522.2 ± 104.5
TCO_2 (µmol kg^{-1})	2518.6 ± 20.9	2704.7 ± 36.9	2424.4 ± 60.3	2558.0 ± 60.0	2354.6 ± 47.9	2678.5 ± 58.6	2348.9 ± 80.6	2681.3 ± 59.5
***HCO_3^-* (µmol kg^{-1})**	2273.5 ± 34.8	2563.3 ± 35.9	2162.4 ± 42.4	2413.9 ± 53.6	2138.4 ± 79.9	2538.5 ± 51.5	2105.2 ± 80.9	2528.2 ± 58.1
CO_3^{2-} (µmol kg^{-1})	229.1 ± 24.7	90.9 ± 4.1	247.7 ± 25.4	100.1 ± 7.3	199.5 ± 40.3	90.0 ± 11.6	228.6 ± 51.1	108.9 ± 4.1
Ω Cal	5.5 ± 0.6	2.2 ± 0.1	5.9 ± 0.6	2.4 ± 0.2	4.8 ± 1.0	2.2 ± 0.3	5.5 ± 1.2	2.6 ± 0.1
Ω Ara	3.5 ± 0.4	1.4 ± 0.1	3.9 ± 0.4	1.6 ± 0.1	3.1 ± 0.6	1.4 ± 0.2	3.6 ± 0.8	1.7 ± 0.1

Table A.8.3. Retention times and quantification ions (m/z) for TCS determination through GC-MS, as well as, validation parameters. Method detection and quantification limits (MDL, MQL) for fish tissues and feed are expressed in ng kg^{-1}, dry weight, whereas for water values are presented in µg L^{-1}.

Analyte	Retention time	SIM ions m/z	Matrix	MDL	MQL	Recovery %	Precision (relative standard deviation, RSD) %
methyl-TCS D-3 (IS2)	18.27	305, 307, 252, 254	**Brain**	0.015	0.025	72	12
			Liver	0.015	0.025	78	10
TCS	19.34	288, 290, 252, 218	**Muscle**	0.009	0.015	89	8
			Feed	0.009	0.015	91	11
Chrysene D-12 (IS1)	22.68	240, 241, 120, 236	**Seawater**	0.007	0.010	102	7

Table A.8.4. Generalized Linear Models (GLM) of TCS tissue concentration between different treatments. In *p-value* column, values in bolt indicate significant differences.

	Estimate	**Std Error**	**Estimate/ Std Error**	***p-value***
Model: GLM (Gamma)				
Response variable: TCS concentration				
Final model term(s): Principal effects of tissue, pH and temperature (Brain as reference)				
(Intercept)	18.52	2.60	7.13	**1.19E-05**
Tissue (Liver)	-12.57	2.73	-4.61	**6.03E-04**
Tissue (Muscle)	-17.96	2.60	-6.91	**1.62E-05**
Temperature	19.94	5.99	3.33	**0.01**
pH	2.53	3.93	0.64	0.53
Tissue (Liver) × Temperature	10.47	7.91	1.32	0.21
Tissue (Muscle) × Temperature	-16.77	6.01	-2.79	**0.02**
Tissue (Liver) × pH	8.90	4.70	1.89	0.08
Tissue (Muscle) × pH	-1.58	3.94	-0.40	0.70
Temperature × pH	-5.91	8.29	-0.71	0.49
Tissue (Liver) × Temperature × pH	-10.15	11.01	-0.92	0.37
Tissue (Muscle) × Temperature × pH	2.36	8.31	0.28	0.78
Final model term(s): Principal effects of tissue, pH and temperature (Liver as reference)				
(Intercept)	5.95	0.83	7.13	**1.19E-05**
Tissue (Brain)	12.57	2.73	4.61	**6.03E-04**
Tissue (Muscle)	-5.39	0.84	-6.43	**3.25E-05**
Temperature	30.41	5.17	5.89	**7.41E-05**
pH	11.44	2.58	4.44	**8.09E-04**
Tissue (Brain) × Temperature	-10.47	7.91	-1.32	0.21
Tissue (Muscle) × Temperature	-27.24	5.19	-5.25	**2.06E-04**
Tissue (Brain) × pH	-8.90	4.70	-1.89	0.08
Tissue (Muscle) × pH	-10.48	2.59	-4.05	**1.61E-03**
Temperature × pH	-16.06	7.24	-2.22	0.05
Tissue (Brain) × Temperature × pH	10.15	11.01	0.92	0.37
Tissue (Muscle) × Temperature × pH	12.50	7.27	1.72	0.11

Table A.8.5. Generalized Linear Models (GLM) of W, TL, K, HSI and BB_{ratio} between different treatments. In *p-value* column, values in bolt indicate significant differences.

	Estimate	Std Error	Estimate/ Std Error	*p-value*
Model: GLMM (Gaussian)				
Random effect: replicate tank				
Final model term(s): Principal effects of TCS exposure, pH and temperature				
Response variable: W				
(Intercept)	4.48	0.41	10.98	**8.52E-10**
TCS exposure	-0.27	0.54	-0.50	0.62
Temperature	1.98	0.54	3.66	**9.84E-04**
pH	0.60	0.54	1.11	0.28
TCS exposure × Temperature	-0.99	0.76	-1.29	0.21
TCS exposure × pH	0.19	0.76	0.24	0.81
Temperature × pH	-1.49	0.76	-1.95	0.06
TCS exposure × Temperature × pH	1.82	1.08	1.68	0.10
Response variable: TL				
(Intercept)	6.48	0.21	30.80	**2.00E-16**
TCS exposure	0.12	0.28	0.43	0.67
Temperature	1.00	0.28	3.61	**1.11E-03**
pH	0.48	0.28	1.73	0.09
TCS exposure × Temperature	-0.54	0.39	-1.38	0.18
TCS exposure × pH	0.10	0.39	0.26	0.80
Temperature × pH	-0.80	0.39	-2.04	0.05
TCS exposure × Temperature × pH	0.92	0.55	1.66	0.11
Response variable: K				
(Intercept)	0.94	0.16	6.01	**1.49E-06**
TCS exposure	0.38	0.22	1.72	0.10
Temperature	-0.03	0.22	-0.14	0.89
pH	0.18	0.22	0.84	0.41
TCS exposure × Temperature	-0.48	0.31	-1.55	0.13
TCS exposure × pH	-0.21	0.31	-0.68	0.50
Temperature × pH	-0.21	0.31	-0.66	0.51
TCS exposure × Temperature × pH	-0.10	0.44	-0.23	0.82
Response variable: HSI				
(Intercept)	1.64	0.07	25.01	**2.00E-16**
TCS exposure	-0.20	0.09	-2.11	**0.04**
Temperature	-0.13	0.09	-1.37	0.18
pH	-0.14	0.09	-1.55	0.13
TCS exposure × Temperature	0.15	0.13	1.11	0.28
TCS exposure × pH	0.08	0.13	0.64	0.53
Temperature × pH	0.12	0.13	0.92	0.36
TCS exposure × Temperature × pH	-0.17	0.19	-0.92	0.37
Response variable: BB_{ratio}				
(Intercept)	0.97	0.12	8.27	**1.91E-09**
TCS exposure	0.15	0.17	0.88	0.39
Temperature	0.15	0.17	0.92	0.37
pH	0.03	0.17	0.19	0.85
TCS exposure × Temperature	-0.23	0.23	-0.96	0.34
TCS exposure × pH	-0.17	0.23	-0.72	0.48
Temperature × pH	-0.04	0.23	-0.18	0.86
TCS exposure × Temperature × pH	-0.01	0.33	-0.02	0.98

Table A.8.6. Generalized Linear Models (GLM) of tissue biomarker responses between different treatments. In *p-value* column, values in bolt indicate significant differences.

	Estimate	Std Error	Estimate/ Std Error	*p-value*
Model: GLMM (Gaussian)				
Random effect: replicate tank				
Final model term(s): Principal effects of TCS exposure, pH and temperature				
Tissue: Muscle				
Variable: CAT activity				
(Intercept)	21.22	1.61	13.18	**3.92E-08**
TCS exposure	11.42	1.94	5.88	**2.03E-06**
Temperature	1.74	1.94	0.90	0.38
pH	3.83	1.94	1.97	0.06
TCS exposure × Temperature	-13.99	2.75	-5.10	**1.83E-05**
TCS exposure × pH	-13.46	2.75	-4.90	**3.15E-05**
Temperature × pH	-4.78	2.75	-1.74	0.09
TCS exposure × Temperature × pH	13.63	3.88	3.51	**1.45E-03**
Variable: SOD activity				
(Intercept)	82.71	0.94	87.98	**2.00E-16**
TCS exposure	1.55	1.33	1.17	0.25
Temperature	7.47	1.33	5.62	**3.31E-06**
pH	-2.12	1.33	-1.60	0.12
TCS exposure × Temperature	-7.30	1.88	-3.88	**4.85E-04**
TCS exposure × pH	5.73	1.88	3.05	**4.61E-03**
Temperature × pH	2.47	1.88	1.31	0.20
TCS exposure × Temperature × pH	-3.82	2.66	-1.44	0.16
Variable: GST activity				
(Intercept)	62.03	3.53	17.56	**6.53E-13**
TCS exposure	-11.47	4.65	-2.47	**0.02**
Temperature	26.93	4.65	5.80	**2.59E-06**
pH	10.29	4.65	2.22	**0.03**
TCS exposure × Temperature	-44.34	6.57	-6.75	**1.89E-07**
TCS exposure × pH	1.49	6.57	0.23	0.82
Temperature × pH	-22.08	6.57	-3.36	**2.16E-03**
TCS exposure × Temperature × pH	38.53	9.29	4.15	**2.60E-04**
Variable: LPO (MDA content)				
(Intercept)	0.01	0.00	6.19	**6.25E-07**
TCS exposure	0.01	0.00	3.39	**1.88E-03**
Temperature	0.00	0.00	2.18	**0.04**
pH	0.00	0.00	-0.44	0.66
TCS exposure × Temperature	-0.01	0.00	-3.24	**2.78E-03**
TCS exposure × pH	0.01	0.00	2.77	**0.01**
Temperature × pH	0.00	0.00	-0.56	0.58
TCS exposure × Temperature × pH	-0.01	0.00	-1.89	0.07
Variable: HSP70/HSC70 content				
(Intercept)	789.73	75.62	10.44	**7.77E-12**
TCS exposure	-352.09	106.95	-3.29	**2.43E-03**
Temperature	-274.56	106.95	-2.57	**0.02**
pH	-25.71	106.95	-0.24	0.81
TCS exposure × Temperature	556.92	151.25	3.68	**8.47E-04**
TCS exposure × pH	-69.03	151.25	-0.46	0.65
Temperature × pH	256.27	151.25	1.69	0.10
TCS exposure × Temperature × pH	433.36	213.90	2.03	0.05
Variable: Ub content				
(Intercept)	19.63	1.46	13.47	**1.81E-11**
TCS exposure	6.85	1.93	3.55	**1.30E-03**
Temperature	-1.55	1.93	-0.80	0.43
pH	-1.98	1.93	-1.03	0.31
TCS exposure × Temperature	7.73	2.73	2.83	**0.01**
TCS exposure × pH	1.49	2.73	0.55	0.59
Temperature × pH	20.40	2.73	7.48	**2.55E-08**
TCS exposure × Temperature × pH	-26.81	3.86	-6.95	**1.05E-07**

Table A.8.6. ***(continuation)*** Generalized Linear Models (GLM) of tissue biomarker responses between different treatments. In *p-value* column, values in bolt indicate significant differences.

	Estimate	Std Error	Estimate/ Std Error	*p-value*
Model: GLMM (Gaussian)				
Random effect: replicate tank				
Final model term(s): Principal effects of TCS exposure, pH and temperature				
Tissue: Brain				
Variable: CAT activity				
(Intercept)	7.23	0.52	13.94	**1.14E-13**
TCS exposure	-1.15	0.73	-1.58	0.12
Temperature	-2.02	0.73	-2.78	**0.01**
pH	-0.97	0.73	-1.34	0.19
TCS exposure × Temperature	2.84	1.03	2.76	**0.01**
TCS exposure × pH	2.40	1.03	2.34	**0.03**
Temperature × pH	2.46	1.03	2.39	**0.02**
TCS exposure × Temperature × pH	-3.32	1.45	-2.29	**0.03**
Variable: SOD activity				
(Intercept)	86.64	1.43	60.60	**<2E-16**
TCS exposure	-3.84	1.99	-1.93	0.06
Temperature	1.92	1.99	0.97	0.34
pH	-0.14	1.99	-0.07	0.94
TCS exposure × Temperature	-1.03	2.81	-0.37	0.72
TCS exposure × pH	-0.82	2.81	-0.29	0.77
Temperature × pH	1.49	2.81	0.53	0.60
TCS exposure × Temperature × pH	-0.36	3.98	-0.09	0.93
Variable: GST activity				
(Intercept)	8.32	1.37	6.09	**8.47E-07**
TCS exposure	1.61	1.93	0.83	0.41
Temperature	12.12	1.93	6.27	**4.96E-07**
pH	6.33	1.93	3.27	**2.56E-03**
TCS exposure × Temperature	-13.01	2.73	-4.76	**4.00E-05**
TCS exposure × pH	-4.44	2.73	-1.63	0.11
Temperature × pH	-7.10	2.73	-2.60	**0.01**
TCS exposure × Temperature × pH	4.67	3.87	1.21	0.24
Variable: LPO (MDA content)				
(Intercept)	0.01	0.00	9.29	**1.35E-10**
TCS exposure	0.00	0.00	0.11	0.91
Temperature	0.00	0.00	-3.92	**4.42E-04**
pH	0.00	0.00	-0.40	0.69
TCS exposure × Temperature	0.00	0.00	3.70	**8.06E-04**
TCS exposure × pH	0.00	0.00	0.03	0.98
Temperature × pH	0.00	0.00	0.37	0.71
TCS exposure × Temperature × pH	0.00	0.00	0.56	0.58
Variable: HSP70/HSC70 content				
(Intercept)	166.80	13.31	12.54	**6.88E-14**
TCS exposure	-37.46	18.82	-1.99	0.06
Temperature	-67.86	18.82	-3.61	**1.04E-03**
pH	-67.51	18.82	-3.59	**1.10E-03**
TCS exposure × Temperature	81.11	26.61	3.05	**4.60E-03**
TCS exposure × pH	64.24	26.61	2.41	**0.02**
Temperature × pH	93.24	26.61	3.50	**1.38E-03**
TCS exposure × Temperature × pH	-120.44	37.64	-3.20	**3.09E-03**
Variable: Ub content				
(Intercept)	22.12	1.57	14.11	**2.16E-09**
TCS exposure	-4.89	1.92	-2.55	**0.02**
Temperature	-10.30	1.92	-5.36	**8.51E-06**
pH	-15.51	1.92	-8.07	**5.27E-09**
TCS exposure × Temperature	6.38	2.72	2.35	**0.03**
TCS exposure × pH	12.71	2.72	4.68	**5.83E-05**
Temperature × pH	21.04	2.72	7.74	**1.23E-08**
TCS exposure × Temperature × pH	-21.44	3.84	-5.58	**4.55E-06**

Table A.8.6. ***(continuation)*** Generalized Linear Models (GLM) of tissue biomarker responses between different treatments. In *p-value* column, values in bolt indicate significant differences.

	Estimate	Std Error	Estimate/ Std Error	*p-value*
Model: GLMM (Gaussian)				
Random effect: replicate tank				
Final model term(s): Principal effects of TCS exposure, pH and temperature				
Tissue: Brain				
Variable: AChE activity				
(Intercept)	1853.71	42.73	43.39	**2.00E-16**
TCS exposure	-427.09	60.42	-7.07	**5.13E-08**
Temperature	-512.37	60.42	-8.48	**1.09E-09**
pH	-84.35	60.42	-1.40	0.17
TCS exposure × Temperature	759.49	85.45	8.89	**3.74E-10**
TCS exposure × pH	469.19	85.45	5.49	**4.76E-06**
Temperature × pH	75.68	85.45	0.89	0.38
TCS exposure × Temperature × pH	-696.03	120.85	-5.76	**2.18E-06**
Tissue: Liver				
Variable: CAT activity				
(Intercept)	17.74	1.42	12.53	**7.00E-14**
TCS exposure	-1.86	2.00	-0.93	0.36
Temperature	0.59	2.00	0.30	0.77
pH	-1.77	2.00	-0.88	0.38
TCS exposure × Temperature	-5.59	2.83	-1.98	0.06
TCS exposure × pH	4.02	2.83	1.42	0.17
Temperature × pH	-2.50	2.83	-0.88	0.38
TCS exposure × Temperature × pH	9.95	4.00	2.49	**0.02**
Variable: SOD activity				
(Intercept)	85.54	1.41	60.81	**2.00E-16**
TCS exposure	-1.06	1.95	-0.54	0.59
Temperature	-0.64	1.95	-0.33	0.75
pH	2.29	1.95	1.18	0.25
TCS exposure × Temperature	4.56	2.75	1.66	0.11
TCS exposure × pH	-17.13	2.75	-6.23	**7.01E-07**
Temperature × pH	1.19	2.75	0.43	0.67
TCS exposure × Temperature × pH	10.94	3.89	2.81	**0.01**
Variable: GST activity				
(Intercept)	101.25	8.76	11.56	**1.86E-10**
TCS exposure	-19.85	11.53	-1.72	0.10
Temperature	61.55	11.53	5.34	**8.80E-06**
pH	28.16	11.53	2.44	**0.02**
TCS exposure × Temperature	-111.83	16.30	-6.86	**1.26E-07**
TCS exposure × pH	39.09	16.30	2.40	**0.02**
Temperature × pH	-89.20	16.30	-5.47	**6.06E-06**
TCS exposure × Temperature × pH	79.69	23.06	3.46	**1.65E-03**
Variable: LPO (MDA content)				
(Intercept)	0.00	0.00	2.29	**0.03**
TCS exposure	0.01	0.00	3.15	**3.53E-03**
Temperature	0.00	0.00	1.26	0.22
pH	0.00	0.00	0.08	0.94
TCS exposure × Temperature	-0.01	0.00	-2.69	**0.01**
TCS exposure × pH	0.02	0.00	4.26	**1.66E-04**
Temperature × pH	0.00	0.00	-0.05	0.96
TCS exposure × Temperature × pH	-0.02	0.01	-3.34	**2.15E-03**
Variable: HSP70/HSC70 content				
(Intercept)	772.26	55.08	14.02	**3.27E-15**
TCS exposure	-158.08	77.89	-2.03	0.05
Temperature	-52.97	77.89	-0.68	0.50
pH	488.23	77.89	6.27	**5.01E-07**
TCS exposure × Temperature	-71.45	110.16	-0.65	0.52
TCS exposure × pH	-918.32	110.16	-8.34	**1.59E-09**
Temperature × pH	-1006.93	110.16	-9.14	**1.95E-10**
TCS exposure × Temperature × pH	1197.45	155.78	7.69	**9.22E-09**

Table A.8.6. ***(continuation)*** Generalized Linear Models (GLM) of tissue biomarker responses between different treatments. In *p-value* column, values in bolt indicate significant differences.

	Estimate	Std Error	Estimate/ Std Error	*p-value*
Model: GLMM (Gaussian)				
Random effect: replicate tank				
Final model term(s): Principal effects of TCS exposure, pH and temperature				
Tissue: Liver				
Variable: Ub content				
(Intercept)	8.41	1.44	5.85	**1.69E-06**
TCS exposure	1.42	2.04	0.70	0.49
Temperature	3.83	2.04	1.88	0.07
pH	9.17	2.04	4.51	**8.32E-05**
TCS exposure × Temperature	-3.92	2.88	-1.36	0.18
TCS exposure × pH	3.24	2.88	1.13	0.27
Temperature × pH	-11.60	2.88	-4.03	**3.21E-04**
TCS exposure × Temperature × pH	19.84	4.07	4.87	**2.87E-05**
Variable: VTG content				
(Intercept)	62.66	6.70	9.35	**1.14E-10**
TCS exposure	-48.58	9.47	-5.13	**1.37E-05**
Temperature	-37.80	9.47	-3.99	**3.60E-04**
pH	15.34	9.47	1.62	0.12
TCS exposure × Temperature	40.80	13.40	3.05	**4.63E-03**
TCS exposure × pH	-13.75	13.40	-1.03	0.31
Temperature × pH	7.80	13.40	0.58	0.56
TCS exposure × Temperature × pH	4.21	18.95	0.22	0.83

Table A.8.7. Summary of significant changes (%) induced by TCS exposure, acidification and warming in relation to the average values obtained in Control treatment. "↑" before the value indicates a significant up-regulation in relation to the Control treatment, whereas "↓" indicates a significant down-regulation ($p < 0.05$). Abbreviations: K - Fulton's condition index; HIS - hepatosomatic index; BB_{ratio} - brain-to-body mass ratio; CAT – catalase; SOD – superoxide dismutase; GST – glutathione S-transferase; LPO - lipid peroxidation; HSP70/HSC70 - heat shock proteins; Ub - total ubiquitin; AChE – acetylcholinesterase; VTG – vitellogenin; NS – No significant alteration ($p > 0.05$) in relation to the Control treatment; TCS – triclosan exposure; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 ºC).

Stressor	Animal Fitness				Tissue molecular responses							
	K	HSI	BB_{ratio}		CAT activity	SOD activity	GST activity	LPO	HSP70/HSC70 content	Ub content	AChE activity	VTG content
Acidification	NS	NS	NS	**Muscle**	NS	NS	NS	NS	NS	NS	-	-
				Liver	NS	NS	NS	NS	↑63.2	↑>100	-	NS
				Brain	NS	NS	↑76.0	NS	NS	↓69.5	NS	-
Warming	NS	NS	NS	**Muscle**	NS	↓9.0	NS	NS	NS	NS	-	-
				Liver	NS	NS	NS	NS	NS	NS	-	↓38.5
				Brain	NS	NS	↑>100	NS	NS	↓46.2	↓27.6	-
Acidification+ Warming	NS	NS	NS	**Muscle**	NS	↓9.4	NS	NS	NS	↑85.4	-	-
				Liver	NS	NS	NS	NS	↓74.0	NS	-	↓68.1
				Brain	NS	NS	↑>100	NS	NS	NS	↓28.1	-
TCS	NS	NS	NS	**Muscle**	↑54.5	NS	NS	NS	↓44.6	NS	-	-
				Liver	NS	NS	NS	↑>100	NS	NS	-	↓79.9
				Brain	NS	NS	NS	NS	NS	NS	↓23.0	-
TCS+ Acidification	NS	NS	NS	**Muscle**	NS	NS	NS	↑>100	↓56.6	NS	-	-
				Liver	NS	↑18.6	NS	↑>100	↓76.2	↑>100	-	↓81.9
				Brain	NS	↑5.5	NS	NS	NS	↓34.5	NS	-
TCS+ Warming	NS	NS	NS	**Muscle**	NS	NS	↓46.8	NS	NS	↑66.0	-	-
				Liver	↓38.7	NS	↓69.6	NS	↓36.6	NS	-	↓60.7
				Brain	NS	NS	NS	NS	NS	↓39.5	NS	-
TCS+Acidification+ Warming	NS	NS	NS	**Muscle**	NS	NS	NS	NS	↑66.5	NS	-	-
				Liver	NS	NS	NS	NS	↓67.6	↑>100	-	↓78.1
				Brain	NS	NS	NS	NS	NS	↓53.9	↓22.4	-

ANNEX 9.

SUPPLEMENTARY INFORMATION FOR CHAPTER 6.

Assessing the effects of seawater temperature and pH on the bioaccumulation of emerging chemical contaminants in marine bivalves

Methodologies

1.1. Quantification of dechloranes 602, 603 and 604 in seawater and bivalve samples

1.1.1. Reagents and chemicals

All reagents and chemicals were of analytical grade or higher. Dec 602 (95 %), Dec 603 (98 %) and Dec 604 (98 %) were purchased from Toronto Research Chemical Inc. (Toronto, ON, Canada). Internal standard 13C-syn-DP was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). Dichloromethane (DCM), hexane and sulphuric acid were purchased from Merck (Darmstadt, Germany). Al-N cartridges were provided by Biotage (Uppsala, Sweden).

1.1.2. Sample preparation

Water samples (9 mL) were spiked with 3.2 ng of 13C-syn-DP. After two hours, the extraction was carried out by ultrasound assisted extraction using 2 mL of hexane, 15 min sonication and 7 min centrifugation at 3500 rpm. The hexane was transferred to a vial. The extract was then reconstituted with 40 µL of toluene for the instrumental analysis.

The extraction of dechloranes from bivalve and feed samples was carried out using a previously optimized method (de la Cal et al., 2003; Labandeira et al., 2007). Freeze-dried sample (1-1.5 g for clams and 0.5 g for feed) was spiked with 3.2 ng of 13C-syn-DP. Pressurized liquid extraction (PLE) was used with hexane:DCM (1:1). Then, the lipid content was determined gravimetrically. The extract was re-dissolved in hexane and fat was removed with concentrated sulphuric acid. The organic phase underwent a solid phase extraction (SPE) using neuter alumina cartridges (5 g) and hexane:DCM (1:2) as elution mixture. Extracts were reconstituted in 40 µL of toluene for the instrumental analysis.

1.1.3. Instrumental analysis

Instrumental analysis was the same for water, biota and feed samples. Extracts were analysed with an Agilent 7890A gas chromatograph coupled to an Agilent 7000B triple quadrupole mass spectrometer according to previous work (Barón et al., 2012). Chromatographic separation was carried out with a DB-5ms column (15 m × 0.25 mm × 0.1 µm of film thickness). For the spectrometric determination negative ion chemical ionization (NICI) at 175 °C was used, with methane as ionisation gas. Selective reaction monitoring (SRM) mode was used with two transitions monitored for each compound. Recoveries for individual compounds ranged 88-99 % and relative standard deviations (RSDs) were 12-22 %.

1.2. Quantification of total (seawater) and inorganic arsenic (bivalve samples)

1.2.1. Reagents and chemicals

All reagents and chemicals were of analytical grade or higher. <18 MΩ cm water was used throughout (Milli-Q-Integral system, Merck, Germany). Nitric acid (PlasmaPure, SCP Science, Courtaboeuf, France) and hydrogenperoxide (Merck, Darmstadt, Germany) were used for sample preparation.

1.2.2. Sample preparation

Determination of inorganic arsenic (iAs) followed the standard (EN 16802:2016) recently issued by European Committee for Standardization (CEN, 2016). For extraction of biota, dilute acid (0.1 M nitric acid) of samples (0.3-0.5 g) and quantitative oxidation of arsenite (iAsIII) to arsenate (iAsV) by hydrogenperoxide (3 % v/v) was applied (Rasmussen et al., 2012).

Water samples were prepared by simple dilution with 2% (v/v) nitric acid prior to analysis of total As (typically x10).

1.2.3. Instrumental analysis

Inorganic arsenic was analysed using anion exchange HPLC (High Performance Liquid Chromatography) (1100 HPLC Agilent Technologies, Waldbronn, Germany) and detected by the on-line coupling to ICP-MS (inductively coupled plasma mass spectrometry) (Agilent 7500ce ICP-MS, Santa Clara, USA) in no gas mode. Total arsenic was determined by the ICP-MS in no-gas mode (m/z 75) with rhodium (m/z 103) as internal standard. For quantification external calibration with matrix-matched standards was used. A blank sample was in all series analyzed in the same conditions as the samples and was subtracted from results prior to reporting.

The accuracy of the methods was evaluated using the certified reference materials DORM-4 (Dogfish muscle) and TORT-2 (Lobster Hepatopancreas) from National Research Council of Canada (Ontario, Canada) for total arsenic [DORM-4: certified = 6.87 ± 0.44 mg kg^{-1}, present work (n = 2) = 6.73 ± 0.22 mg kg^{-1}; TORT-2: certified = 21.6 ± 1.8 mg kg^{-1}, present work (n = 2) = 19.52 ± 0.56 mg kg^{-1}], and ERM-BC211 (rice) from Institute of Reference Materials and Measurements (Geel, Belgium) and NMIJ CRM 7405-a (marine algae Hijiki) from National Metrology Institute of Japan (NMIJ; Tsukuba, Japan) for iAs [ERM-BC211: 0.124 ± 0.011 mg kg^{-1}, present work (n = 1) = 0.150 mg kg^{-1}; NMIJ 7405-a: 10.1 ± 0.5 mg kg^{-1}, present work (n = 1) = 9.65 mg kg^{-1}].

1.3. Quantification of TBBPA in seawater and bivalve samples

1.3.1. Reagents and chemicals

All reagents and chemicals were of analytical grade or higher. Tetrabromobisphenol A (TBBPA; 99 % purity) and tetrabromobisphenol A ring-13C12 (TBBPA13C12; 99 % purity) used as internal standard (I.S.) were purchased from Sigma-Aldrich (West Chester, PA, USA). Individual standard solution of the TBBPA was prepared in methanol (MeOH, HPLC grade from Sigma-Aldrich) at concentration of 2000 μg L^{-1}. QuEChERS solvents acetonitrile (MeCN, gradient grade for HPLC; 78.6 % purity) and anhydrous magnesium sulfate (anhydrous $MgSO_4$; 99.5 % purity) were purchased from Sigma-Aldrich; formic acid, hydrochloric acid (HCl), sodium chloride (NaCl; 99.5 % purity), and ammonium acetate (97 % purity) were purchased from AppliChem Panreac ITW Co. (Barcelona, Spain). LLE solvents n-hexane (gradient grade for HPLC), tert-butyl methyl ether (MTBE, pro-analysis), and benzene (pro-analysis) were purchased from Merck (Darmstadt, Germany). Formic acid (99%) and chloride acid were purchased VWR Int. (Radnor, PA, USA). Ultra-pure Milli-Q water was obtained using a Millipore Milli-Q system (Millipore, Bedford, MA, USA).

1.3.2. Sample preparation

TBBPA in bivalve samples was extracted using the following procedure based on QuEChERS – LLE extraction, which was previously described in more detail (Cunha et al., 2017). Briefly, 2 g of thoroughly homogenized sample were weighed into a 40 mL glass vial tube, and 80 μL of TBBPA13C12 at 1000 ng mL^{-1} (I.S. working solution) were added. Then, 7 mL of ultra-pure MilliQ water were added, and sealed tubes were handshaked it for 2 min. 10 mL of MeCN, 100 μL of HCl 10M (pH = 4.0 units), 4 g of anhydrous $MgSO_4$ and 1 g of NaCl were added. Sealed tubes were shaken again vigorously by hand for 10 min. Extracts were centrifuged at 2000 g for 5 min. Then, a liquid-liquid extraction procedure was performed, by extracting 3 mL of MeCN extracts to a 15 mL glass vial tube with 7 mL of ultra-pure MilliQ water, adding 4 mL of n-hexane:MTBE (3:1, v/v), and handshaking vigorously again. Subsequently, 3 mL of the upper layer were extracted to a new 15 mL vials, and add 4 mL of nhexane:benzene (3:1, v/v) were added, followed by handshaking. Once again, 3 mL of the upper layer were transferred to 15 mL glass vials. The final extracts were concentrated under a stream of nitrogen (SBH CONC/1 sample concentrator from Stuart®, Staffordshire, OSA, USA), reconstituted in mobile phase - 100μL of 5mM ammonium acetate and 900 μL of MeOH, and transferred to a 2 mL glass vials before LC-MS/MS analysis. The same procedure was carried out for water samples, using 2 g of each sample.

1.3.3. Instrumental analysis

TBBPA was analysed in seawater by direct injection on LC-MS/MS with prior addition of 80 µL of TBBPA13C12 (IS, 1000 µg L^{-1}). Bivalve and feed sample extracts were analyzed by LC-MS/MS, i.e. a high-performance liquid chromatography (HPLC) system Waters Alliance 2695 (Waters, Milford, MA, USA) interfaced to a Quattro Micro triple quadrupole mass spectrometer (Waters, Manchester, UK). Chromatographic separation was achieved using a Kinetex C18 2.6 µ particle size analytical column (150 × 4.6 mm) with pre-column from Phenomenex (Tecnocroma, Portugal), at a flow-rate of 200 µL min^{-1}. The optimized MS/MS parameters for the target analytes are described in Cunha et al. (2017).

For quality control purposes, matrix-matched calibration curves were achieved by analyzing blank samples (free of TBBPA) spiked with known amounts of the analytes. Analytes concentration in the analyzed samples was obtained by the I.S. method.

1.4. Quantification of PFOA and PFOS in seawater and bivalve samples

1.4.1. Reagents and chemicals

All reagents and chemicals were of analytical grade or higher. Acetonitrile, acetone, n-hexane, hydrochloric acid, sodium hydroxide and methanol were purchased from LGC Standards (Wesel, Germany). Ammonium formiate and ENVIcarb were purchased from Sigma-Aldrich (Zwijndrecht, Netherlands). Native and 13C4 labelled PFOS and PFOA were purchased from Wellington Laboratories (Guelph, Canada). Oasis HLB SPE cartridges were purchased from Waters (Etten-Leur, the Netherlands). Sodium sulfate was dried for 20 hours at 450 °C before use. Internal solutions consisted of 150 ng mL^{-1} 13C4-PFOS and 13C4-PFOA in acetonitrile.

1.4.2. Sample preparation

PFOS and PFOA were extracted and analysed using the methods previously described in Kwadijk et al. (2010). For water samples, 350 µL of internal standard solution was added to 10 mL samples. HLB cartridges were activated using 10 mL of acetonitrile followed by 10 mL of demi water after which the sample was introduced. Then, 10 mL of acetonitrile was used to elute the sample. Extracts were dried using sodium sulphate and cleaned with ENVIcarb after which they were transferred to a vial for analysis.

For bivalves, 5 g of sample were weighed in a 15 mL polypropylene (pp) tube and subsequently extracted 3 times using acetonitrile. The extract was dried over a filter with sodium sulphate and concentrated to 10 mL. The extract was washed 3 times using 10 mL of hexane after which the extract was concentrated to 0.7 mL and transferred to a centrifuge tube containing 50 mg ENVIcarb. The tube was mixed and subsequently centrifuged. The extract was transferred to a vial for analysis.

1.4.3. Instrumental analysis

Instrumental analysis was performed using a Thermo Finnigan (Waltham, MA) Surveyor Autosampler and an HPLC system coupled with a Thermo Finnigan LCQ advantage Ion-Trap MS instrument with electrospray (ESI-MS/MS). Separation was performed by injecting 20 µL of extract onto a 100 x 2.10 mm (5µm) Fluorphase RP column using ammonium formate and formic acid in acetonitrile as mobile phase A and ammonium formate and formic acid in demi water as mobile phase B. Quantification was performed in ESI negative mode monitoring m/z 499 for PFOS, 503 for 13C-PFOS, 413-->369 for PFOA and 417 -->372 for 13C-PFOA.

For quality control purposes, an internal reference sample was analysed along with each set of samples. Results were satisfactory for water, as well as biota samples. PFOS and PFOA were < LOD in blank samples (< 0.3 µg kg^{-1} for biota and <0.03µg L^{-1} for water).

TABLES

Table A.9.1. Control and contaminated feeds' composition.

Ingredients	%
Micronized fishmeal LT70[1]	35.0
Fish protein concentrate[2]	35.0
Squid meal[3]	10.0
Wheat gluten[4]	5.0
Soy lecithin[5]	11.0
Vitamin and mineral premix[6]	4.0

[1] MicroNorse: 72 % crude protein (CP), 11% crude fat (CF), Tromsø Fiskeindustri A/S,Norway.
[2] CPSP 90: 84 % CP, 12 % CF, Sopropêche, France.
[3] Super prime without guts: 82% CP, 3.5% CF, Sopropêche, France;
[4] VITAL: 85.7 % CP, 1.3 % CF, ROQUETTE, France.
[5] LECICO P 700IPM, LECICO GmbH, Germany.
[6] PREMIX Lda, Portugal: Vitamins (IU or mg kg^{-1} diet): DL-alpha tocopherol acetate, 400 mg; sodium menadione bisulphate, 100 mg; retinyl acetate, 80000 IU; DL-cholecalciferol, 8000 IU; thiamin, 120 mg; riboflavin, 120 mg; pyridoxine, 80 mg; cyanocobalamin, 0.4 mg; nicotinic acid, 800 mg; folic acid, 60 mg; ascorbic acid, 2000 mg; inositol, 2000 mg; biotin, 12 mg; calcium panthotenate, 400 mg; choline chloride, 4000 mg, betaine, 2000 mg. Minerals (g or mg kg^{-1} diet): copper sulphate, 36 mg; ferric sulphate, 24 mg; potassium iodide, 2 mg; manganese oxide, 38.4 mg; sodium selenite, 0.04 mg; zinc sulphate, 30 mg; sodium chloride, 1600 mg; excipient wheat middlings.

Table A.9.2. Seawater physical and chemical parameters (mean ± standard deviation) in each treatment of Trials I and II. Abbreviations: CTR – control (non-contaminated), 19 ºC, 8.0 pH units; CONT - contaminated, 19 ºC, 8.0 pH units; CONT+Acid - contaminated, 19 ºC, 7.6 pH units; CONT+Warm - contaminated, 23 ºC, 8.0 pH units; CONT+Acid+Warm - contaminated, 23 ºC, 7.6 pH units; TA – total alkalinity; P CO_2 - partial CO_2 pressure; T CO_2 - Total CO_2 concentration; HCO_3^- - bicarbonate; CO_3^{2-} - carbonate ion concentrations; ΩCal - calcite saturation state; ΩAra – aragonite saturation state.

Trial I	CTR	CONT	CONT+ Acid	CONT+ Warm	CONT+Acid+ Warm
Temp (°C)	19.0 ± 0.2	19.0 ± 0.2	19.0 ± 0.2	23.0 ± 0.2	23.0 ± 0.2
Salinity (‰)	34.8 ± 0.8	35.2 ± 0.9	35.1 ± 0.5	34.9 ± 0.6	34.8 ± 0.9
pH (units)	8.01 ± 0.01	8.04 ± 0.03	7.62 ± 0.03	8.01 ± 0.04	7.64 ± 0.04
TA (µmol kg^{-1})	2022.3 ± 110.1	2051.9 ± 80.2	1982.4 ± 100.1	2029.4 ± 90.1	1938.7 ± 122.1
P CO_2 (µatm)	386.5 ± 7.5	392.1 ± 4.7	1079.0 ± 56.9	387.9 ± 6.1	1048.5 ± 62.1
T CO_2 (µmol kg^{-1})	1805.5 ± 34.7	1823.7 ± 23.8	1907.9 ± 49.9	1812.4 ± 61.3	1873.3 ± 56.9
HCO_3^- (µmol kg^{-1})	1643.2 ± 110.3	1653.2 ± 99.8	1804.2 ± 130.5	1649.7 ± 111.9	1774.8 ± 143.6
CO_3^{2-} (µmol kg^{-1})	149.7 ± 33.6	158.1 ± 24.5	70.4 ± 6.3	150.0 ± 29.9	64.3 ± 9.9
Ω Ara	2.3 ± 0.4	2.5 ± 0.2	1.1 ± 0.1	2.3 ± 0.4	1.0 ± 0.2
Ω Cal	3.6 ± 0.5	3.8 ± 0.3	1.7 ± 0.6	3.6 ± 0.1	1.5 ± 0.7
Trial II					
Temp (°C)	19.0 ± 0.2	19.0 ± 0.3	19.0 ± 0.1	23.0 ± 0.1	23.0 ± 0.2
Salinity (‰)	35.1 ± 0.7	35.2 ± 0.6	35.1 ± 0.7	34.8 ± 0.8	34.9 ± 0.6
pH (units)	8.01 ± 0.02	8.01 ± 0.03	7.63 ± 0.02	8.02 ± 0.03	7.62 ± 0.02
TA (µmol kg^{-1})	2112.4 ± 111.1	2141.9 ± 100.2	2002.4 ± 120.1	2130.8 ± 100.3	1998.7 ± 90.9
P CO_2 (µatm)	404.5 ± 7.9	410.0 ± 6.8	1090.1 ± 99.8	408.1 ± 5.2	1081.5 ± 80.4
T CO_2 (µmol kg^{-1})	1889.2 ± 39.8	1907.0 ± 21.1	1927.5 ± 43.8	1906.7 ± 23.9	1932.3 ± 38.7
HCO_3^- (µmol kg^{-1})	1719.3 ± 100.8	1728.8 ± 99.3	1822.7 ± 110.3	1735.5 ± 92.4	1830.8 ± 75.6
CO_3^{2-} (µmol kg^{-1})	156.7 ± 37.2	165.3 ± 23.4	71.1 ± 13.7	157.8 ± 28.9	66.3 ± 10.8
Ω Ara	2.4 ± 0.4	2.6 ± 0.2	1.1 ± 0.5	2.4 ± 0.3	1.1 ± 0.1
Ω Cal	3.7 ± 0.5	3.9 ± 0.2	1.7 ± 0.4	3.8 ± 0.2	1.6 ± 0.2