OCCURRENCE, FATE AND EFFECTS OF AZOXYSTROBIN IN AQUATIC ECOSYSTEMS

Elsa Teresa Santos Rodrigues
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STATEMENT OF ORIGINALITY

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The chapters of this thesis have been written as papers for international peer-reviewed journals. The current publication status of the papers is presented in the front page of each Chapter. All these papers have been reworked so that they are presented in a consistent style and format in this dissertation. For those papers which have been published, copyright rests with the publishers. Since this project began four years ago, the bibliography presented here was updated, being the changes written in grey colour.

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Date, 20 de janeiro de 2016
STATEMENT OF ETHICS

All animal experiments were conducted in accordance with the ethical guidelines of the European Union Council (Directive 2010/63/EU) and the Portuguese Agricultural Ministry (Decreto-Lei 113/2013) for the protection of animals used for experimental and other scientific purposes. I, Elsa Teresa Santos Rodrigues, am accredited for the use of live animals for scientific purposes (category C) according to the Federation of European Laboratory Animal Science Associations (FELASA) education and training guidelines, granted by the Portuguese General Directorate of Veterinary.

Signature,

Date, 20 de janeiro de 2016
PREFACE

The study project underlying this thesis was based on the peer review of the pesticide risk assessment of the active substance azoxystrobin reported by the European Food Safety Authority (EFSA) in 2010. Azoxystrobin is currently the world’s leading agricultural fungicide and, at that time, the mentioned document concluded that this risk assessment addressed all non-target species except aquatic organisms. Therefore, a comprehensive project was designed in order to contribute and timely respond to this critical area of concern.

The project started with a scientific review paper aiming to assess current azoxystrobin environmental exposure and compile relevant toxicity data. At the time, the mentioned review concluded, among other things, that important knowledge regarding base-line values of azoxystrobin in natural marine ecosystems were lacking and that there were very few data on toxicity to different aquatic organisms, especially in what concerns marine organisms. Also, validated analytical methods for the measurement of azoxystrobin in complex matrices such as sediment, algae, aquatic plants and aquatic animals were very limited. Hence, in order to contribute to the filling of these gaps, an estuarine area was chosen as a field case study, and the experimental model organisms used in the toxicity tests were all marine species. In this project, “marine species” include those found in estuarine, coastal, and open ocean habitats. In addition, adequate analytical methodologies for pesticide multi-residue determination were developed and validated for complex aquatic matrices (sediment, macrophyte and animal samples), being these cutting-edge research methodologies in compliance with Green Chemistry principles.
Pesticides particularly adversely affect estuaries as they are usually adjacent to large cultivated areas (river valleys), and most pesticide discharges, leaching and runoff processes flow into such systems. The Mondego estuary was chosen as a field case study for it presents several anthropogenic problems due, among other factors, to about 12,300 ha of upstream intensively cultivated land (mainly maize and rice crops). The seasonal and spatial occurrence and fate of legacy and current-use pesticides were studied in this estuary. Therefore, pesticide residues were prospected both in water and in complex matrices such as sediment, macroalgae, aquatic plants, aquatic worms and bivalves. Pesticide extraction and clean-up multi-residue analytical method by Selective Pressurised Liquid Extraction (SPLE) followed by on-line Solid Phase Extraction and Ultra Performance Liquid Chromatography-tandem Mass Spectrometry (on-line SPE-UPLC-MS/MS) was the methodology used for the chemical determinations.

The ecotoxicological strategy has as its origin a tiered testing concept where a hierarchy of tests reflecting the increasing complexity of biological organisation, plus the estimation of environmental exposure are used to predict potential environmental hazard. Based on this premise, the toxicity tests performed in this project addressed mitochondrial toxicity assessment (subcellular level approach), cell-based assays (cellular level approach), single-species toxicity tests (population level approach), as well as the use of the Species Sensitivity Distribution (SSD) concept to assess the community level approach. A second point of interest was then added to these tasks. For instance, in the subcellular level approach, the effect of temperature as a physical factor was considered as well, for in addition to the considerable stress caused by environmental pollutants, currently, estuaries may also have to tackle an increase in temperature caused by climate-change. Therefore, a multi-stress experimental design was developed to assess the ecological effect of azoxystrobin under extreme temperatures. In the population and
community level approaches, the study intended to contribute with novel information on the effects of short-term azoxystrobin exposures on marine organisms. Furthermore, this study also considered the noxious potential of the azoxystrobin commercial formulation Ortiva®, and related the toxicity marine data with the toxicity freshwater data reported in literature. Commercial formulations of pesticides often contain inert ingredients in addition to their active ingredients. However, information on what inert ingredients are included in the formulations is not usually provided, nor are their potential effects.

In a transversal way this project also reflects ethic concerns related to the protection of animals used for scientific purposes, which is the basis of the current European legislation. Modern laboratory animal science was assumedly built on the principle of the 3 Rs, with the aim to reduce the number of animals in ecotoxicology testing, refine or limit the pain and distress to which animals are exposed, and replace the use of animals with non-animal alternatives whenever possible. Hence, the development of alternative assays became an important topic of this project, and therefore it included the use of lower organisms as surrogates for vertebrates (Chapter III used the crab Carcinus maenas as a model organism), as well as of alternative life stages of fish (Chapter IV and V used juveniles and larvae of fish, respectively), and in vitro tests (Chapter IV described cell-based assays as a realible alternative assay to be used in ecotoxicology). Moreover, an extensive review was performed to assess the overall suitability of the invertebrate C. maenas as a routine ecotoxicological test species. Concerning in vitro alternative assays, a step forward was taken by this project. It is known that the replacement of in vivo by in vitro assays can only take place if high-level correlations are found between the results of both tests and if the risk of false negatives is low, so as to maintain the degree of environmental protection. Therefore, an experimental study was developed with the aim to find a sensitive cell model, fish or mammal derived, which
may decrease the risk of false negatives, and after exposing H9c2, a mammalian cell line, to azoxystrobin, a remarkable LC$_{50,96h}$/IC$_{50,72h}$ ratio of 0.998 was found.

Finally, in an integrative conclusion, a preliminary characterisation of azoxystrobin environmental risk was attempted for the aquatic environment.
LIST OF ACRONYMS AND ABBREVIATIONS

6PGDH, 6-phosphogluconate dehydrogenase
ΔΨm, inner mitochondrial membrane potential
AChE, acetylcholinesterase
ADI, acceptable daily intake
ADP, adenosine diphosphate
AF, assessment factor approach
a.i., active ingredient
ANOVA, analysis of variance
AOEL, acceptable operator exposure level
ARfD, acute reference dose
ATP, adenosine triphosphate
AWP, apparent water permeability
AZX, azoxystrobin experimental test 
(Chapter III)
BCF, bioconcentration factor
BChE, butyrylcholinesterase
BMF, biomagnification factor
BP, benzopyrene
BPH, benzopyrene hydroxylase
BPMO, benzopyrene monoxygenase
BRI, biomarker response index
BSA, bovine serum albumin
C, control experimental test 
(Chapter III)
CA, chemical Abstracts index
CAS, chemical Abstracts Service
CAT, catalase
CBE, carboxylesterase
CDNB, 1-chloro-2,4-dinitrobenzene
ChE, cholinesterase
Cl, condition index
CIPAC, collaborative international pesticide analytical council
CV, coefficient of variation of the mean
CYPs, cytochrome P450 enzymes
CYP1A, cytochrome P4501A
d, day or days
DBF, dibenzyllfluorescein dealkylase
DDTs, dichlorodiphenyltrichloroethanes
DMEM, Dulbecco's modified Eagle's medium
DMSO, dimethyl sulphoxide
DNA, deoxyribonucleic acid
DT$_{25}$, time to 25% dissipation
DT$_{50}$, time to 50% dissipation
dw, dry weight
EbC$_{50}$, concentration at which 50% reduction of biomass is observed
EC$_{50}$, median effect concentration
EDTA, ethylenediaminetetraacetic acid
EFSA, European food safety authority
E.g., for example (exempli gratia, Latin)
ELISA, enzyme-linked immunosorbent assay
ERA, environmental risk assessment
ErC$_{50}$, concentration at which 50% growth inhibition is observed
EROD, ethoxyresorufin-O-deethylase
ESI, electrospray ionisation
EU, European Union
FAO, food and agriculture organization of the United Nations
FBS, foetal bovine serum
FCCP, carbonylcyanide p-trifluoromethoxyphenyl-hydrazone
FOCUS, forum for the co-ordination of pesticide fate models and their use
G$_6$PDH, glucose-6-phosphate dehydrogenase
Gill-SI, gill somatic index
G/M, glutamate/malate 
(Chapter III)
GPx, glutathione peroxidase
GR, glutathione reductase
GSH, reduced glutathione
GSI, gonadosomatic index
GSSG, oxidized glutathione
GST, glutathione S-transferase
h, hour or hours
HC₅, hazardous concentration to 1% of the tested taxa
HC₅₅, hazardous concentration to 5% of the tested taxa
HCHs, hexachlorocyclohexane isomers
Hm, haemolymph
Hp, hepatopancreas
HPLC, high performance liquid chromatography
HSI, hepatosomatic index
HSP, stress proteins
IBR, integrated biomarker response
IC₅₀, median inhibition concentration
IDH, NADP⁺-dependent isocitrate dehydrogenase
i.e., that is (id est, Latin)
IGFBP₁, insulin-like growth factor binding protein 1
ISO, international organization for standardization
IUPAC, international union of pure and applied chemistry
Kd, distribution coefficient
Koc, carbon-water partitioning coefficient
Kow, octanol-water partition coefficient
LC₅₀, median lethal concentration
LDH, lactate dehydrogenase
LLHC₅₅, lower-limit HC₅
LMS, lysosomal membrane stability
LPO, lipid peroxidation
LR, linear range
LT₅₀, lethal time for 50% of exposed organisms
MAPK1, mitogen-activated protein kinase 1
MarMAT, marine macroalgae assessment tool
MATC, maximum acceptable toxicant concentrations
MDA, malondialdehyde
MDL, method detection limit
ME, matrix effect
MEC, measured environmental concentration
MeHg, methyl mercury
MeOH, methanol
MFB, multispecies freshwater biomonitor™
MFO, mixed-function oxidase system
min., minute or minutes
MN, micronucleus assay
MQL, method quantification limit
MRL, maximum residue limit
MRM, multiple-reaction monitoring
MS, mass spectrometry
MTs, metallothioneins
NADH, reduced form of nicotinamide adenine dinucleotide
NADH-CcR, NADH cytochrome c reductase
NADH-FR, NADH ferricyanide reductase
NADPH-CcR, NADPH cytochrome c reductase
No., number
NOEAEC, no observed ecologically adverse effect concentration
NOEC, no observable effect concentration
OC, osmoregulatory capacity
OECD, organisation for economic co-operation and development
On-line SPE-UPLC-MS/MS, on-line solid phase extraction and ultra performance liquid chromatography-tandem mass spectrometry
OPP, EPA office of pesticide programs
p.a., pro analysis
PAHs, polycyclic aromatic hydrocarbons
PBDEs, polybrominated diphenyl ethers
PBS, phosphate buffered saline
PCBs, polychlorobiphenyls
pCO₂, carbon dioxide partial pressure
PEC, predicted environmental concentration
PHAG, phagocytic activity
PMRA, health Canada's pest management regulatory agency
PMT, photomultiplier tube
PNEC, Predicted No-Effect-Concentration
POPs, persistent organic pollutants
PORPHY, porphyrins proteins
PSA, primary-secondary amine
PTFE, polytetrafluoroethylene material
PYR, pyrene
Qo, quinol oxidation site
R, extraction recovery
RAC, regulatory acceptable concentration
ROS, reactive oxygen species
RQ, risk quotient
RSD, intra-day precision
RT, retention time
SC, solvent control experimental test (Chapter III)
SD, standard deviation
SFG, scope for growth index
SIM, selected ion monitoring
SOD, superoxide dismutase
SPE, solid phase extraction
SPLE, selective pressurised liquid extraction
SRB, sulforhodamine B
SSD, species sensitivity distribution
TG, total glutathione
TMRE, tetramethylrhodamine ethyl ester perchlorate
TNFR, tumor necrosis factor receptor
TP, total protein
Tris, 2-amino-2-hydroxymethyl-propane-1,3-diol
TU, toxic units
UPLC-MS/MS, ultra performance liquid chromatography-tandem mass spectrometry
US, United States
US-EPA, US environmental protection agency
UV, ultra-violet (detection or detector)
v/v, volume of solute (ml)/volume of solution (ml)
w, week or weeks
WFD, water framework directive
ww, wet weight
ww/dw, wet weight/dry weight ratio
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SUMMARY

After a literature review to find relevant research on the occurrence, fate and effects of azoxystrobin, the world’s leading agricultural fungicide, in aquatic ecosystems, strengths and gaps were identified in the database. Data revealed that validated analytical methods for complex matrices are very limited and knowledge of base-line values of azoxystrobin in marine ecosystems is lacking. The review also showed that there are very few data on azoxystrobin toxicity to different aquatic organisms, especially in what concerns marine organisms. Further work is also required regarding the effects of exposure to multi-stressors.

To successfully determine pesticide levels in sediment, macrophytes and aquatic animals, adequate analytical methodologies were developed and validated. The established methodology applies Selective Pressurised Liquid Extraction (SPLE) followed by on-line solid phase extraction and Ultra Performance Liquid Chromatography-tandem Mass Spectrometry (on-line SPE-UPLC-MS/MS). This cutting-edge research methodology uses a small amount of sample, is time saving and reduces the use of organic solvents, in compliance with Green Chemistry principles.

The seasonal and spatial occurrence and fate of the pesticides atrazine, azoxystrobin, bentazon, λ-cyhalothrin, penoxsulam and terbuthylazine were investigated in the Mondego estuary (Portugal). Quantified concentrations were determined mostly during summer. Azoxystrobin presented the highest detection frequency and atrazine the second highest frequency. Bentazon concentrations in
surface water were considerably higher than those reported for other countries. All the prospected pesticides were bioaccumulated by the bivalve *Scrobicularia plana* and s-triazine pesticides were measured in both seaweeds *Ulva* spp. and *Gracilaria gracilis*. Acknowledging these data, developing and establishing allowable pesticide tolerance values for edible marine species is recommended.

The present project reviewed more than four decades of published research papers in which the crab *Carcinus maenas* was used as an experimental test organism. This survey indicates that *Carcinus* sp. is sensitive to a wide range of aquatic pollutants and that its biological responses are linked to exposure concentrations or doses. Current scientific knowledge regarding the biology and ecology of *Carcinus* sp. and the extensive studies on ecotoxicology found for the present review recognise this crab as a reliable marine model for routine testing in ecotoxicology research and environmental quality assessment, especially in what concerns the application of the biomarker approach.

An ecologically relevant study was conducted to evaluate the responses of *C. maenas* to temperature and azoxystrobin. Superoxide dismutase and glutathione S-transferase activities, mitochondrial oxygen consumption rates and protein content, as well as the Coupling Index were determined. Results provided evidence that crabs' responses to cope with low temperatures were more effective than their responses to cope with high temperatures, which are expected in future climate projections. Moreover, crabs are capable of handling environmental concentrations of azoxystrobin. However, the Coupling Index showed that combined stress factors unbalance crabs' natural capability to handle a single stressor.

The present project also aimed at identifying, among six cell lines, a sensitive cell line whose azoxystrobin *in vitro* median inhibitory concentration (IC$_{50}$) better
matches the azoxystrobin in vivo short-term Sparus aurata median lethal concentration (LC50). Identical absolute sensitivities were attained for both in vitro and in vivo assays for H9c2 cells by the sulforhodamine B colorimetric assay. We concluded that this H9c2 cell-based assay is reliable and represents a suitable ethical alternative to conventional fish assays for azoxystrobin.

This study determined and validated an aquatic Predicted No-Effect-Concentration (PNEC) value for azoxystrobin. The assessment factor and species sensitive distribution approaches were applied to freshwater and marine toxicity datasets. After comparing the PNEC values estimated in the present study to all the laboratory-derived toxicity information available for azoxystrobin, PNEC values derived using the assessment factor method were considered overprotective and a PNEC of 1.0 µg L⁻¹ was recommended for azoxystrobin in the aquatic environmental compartment.

Finally, azoxystrobin environmental risk characterisation could be determined as the Risk Quotient (RQ), the ratio of measured environmental concentrations and its PNEC value. Therefore, since the maximum concentration of azoxystrobin measured in the Mondego estuary was 0.07 µg L⁻¹, it is possible to conclude that here this pesticide currently poses low risk to aquatic organisms (RQ <0.1). Moreover, in general, azoxystrobin may pose a moderate risk to aquatic environments, since most RQ values determined were within the 0.1 and 1.0 range.

KEY-WORDS

Azoxystrobin; Aquatic ecotoxicology; Alternative assays; Mitochondria; Cell-based assays; Species sensitivity distribution.
RESUMO

Depois de feita uma revisão da literatura no que concerne a ocorrência, o destino e os efeitos do pesticida azoxistrobina, líder mundial de fungicidas agrícolas, nos sistemas aquáticos, foi possível identificar lacunas e fragilidades que revelaram ser necessário desenvolver métodos analíticos validados para a sua determinação em matrizes aquáticas complexas. Esta revisão mostrou ainda que a determinação da concentração deste pesticida em sistemas marinhos é quase inexistente e que, no âmbito destes sistemas, existem poucos dados ecotoxicológicos. Mais estudos sobre os efeitos de “stresses” múltiplos são também necessários.

De modo a determinar os níveis de vários pesticidas em sedimento, macrófitas e organismos aquáticos, foram desenvolvidas e validadas metodologias analíticas que usam extracção selectiva por pressurização líquida, seguida de cromatografia líquida de ultra performance associada a espectrometria de massa de alta resolução. Estas metodologias utilizam uma quantidade reduzida de amostra e permitem reduzir o uso de solventes orgânicos, indo ao encontro dos princípios da Química Verde.

As ocorrências sazonal e espacial, assim como o destino dos pesticidas atrazina, azoxistrobina, bentazona, λ-cialotrina, penoxsulame e terbultilazina foram estudados no estuário do Rio Mondego (Portugal). As concentrações quantificáveis foram essencialmente medidas no verão. A azoxistrobina foi o pesticida encontrado com maior frequência, seguido da atrazina. As concentrações de bentazona
medidas na água superficial foram consideravelmente altas quando comparadas com as encontradas em outros países. Todos os pesticidas foram medidos no bivalve *Scrobicularia plana* e os pesticidas s-triazinas em *Ulva* spp. e *Gracilaria gracilis*. Recomenda-se, assim, o estabelecimento de valores limite para espécies marinhas comestíveis.

Este projeto fez uma revisão que representa mais de uma década de utilização do caranguejo *Carcinus maenas* como modelo experimental em ensaios ecotoxicolóxicos. Os resultados evidenciam que o *Carcinus* sp. é sensível a poluentes aquáticos e que as suas respostas estão ligadas à concentração ou à dose utilizada. A exaustiva informação existente sobre a sua biologia e ecologia, assim como a bibliografia encontrada sobre o seu uso em ecotoxicologia, permitem concluir que este caranguejo pode ser considerado um modelo experimental credível, especialmente no que diz respeito à sua utilização em trabalhos com biomarcadores.

Pretendeu-se ainda avaliar as respostas bioquímicas e fisiológicas do *C. maenas* à temperatura e a uma concentração ecologicamente relevante de azoxistrobina. Foram avaliados a actividade das enzimas superoxido dismutase e glutatonia S-transferase, as taxas respiratórias mitocondriais, e o índice de acoplamento mitocondrial. Os resultados indicam que o caranguejo consegue fazer face a temperaturas baixas promovendo alterações bioquímicas e fisiológicas, mas que estas respostas são menos efetivas quando sujeito a temperaturas elevadas. Concluiu-se também que o *C. maenas* consegue adaptar-se à concentração ambiental de azoxistrobina testada. O índice de acoplamento mostrou que a combinação de perturbações diminui a capacidade de o *C. maenas* superar um só “stress”.


Este projeto identificou, de entre seis linhas celulares, aquela que mostra maior sensibilidade, de modo a aproximar a concentração que inibe 50% da proliferação celular (IC_{50}) com a que provoca a morte a 50% dos peixes depois de se efetuar o teste letal com douradas Sparus aurata (LC_{50}). Assim, foi possível identificar um ensaio in vitro igualmente sensível ao ensaio in vivo com peixes. Deste modo, os ensaios com as H9c2 produziram resultados credíveis, podendo ser considerados uma alternativa ecotoxicológica com grandes vantagens éticas.

Pretendeu-se ainda determinar e validar a Concentração sem Efeitos Previsíveis (PNEC) para a azoxistrobina para o meio aquático. Duas abordagens foram utilizadas, uma aplicando um factor de avaliação e outra usando a técnica da distribuição da sensibilidade das espécies. Depois de comparar os seis PNEC obtidos com os resultados de todos os trabalhos existentes na literatura sobre a azoxistrobina, foi possível concluir que a abordagem factor de avaliação é demasiado protetora, tendo sido validado um PNEC de 1.0 \mu g L^{-1}.

Por último, a caracterização do risco ecológico da azoxistrobina nos sistemas aquáticos pode ser feita calculando a razão de um valor de exposição pelo valor PNEC (quociente de risco (RQ)). Uma vez que a concentração máxima obtida para a azoxistrobina medida na água superficial do estuário do Mondego foi de 0.07 \mu g L^{-1}, podemos concluir que este pesticida, neste estuário, coloca baixo risco para os organismos aquáticos e para o meio (RQ <0.1). Podemos ainda concluir que, em geral, a azoxistrobina coloca um risco moderado nos sistemas aquáticos, uma vez que a maioria dos valores RQ obtidos se encontra entre 0.1 e 1.0.

PALAVRAS-CHAVE

Azoxistrobina; Ecotoxicologia aquática; Ensaios alternativos; Mitocôndrias; Ensaios com linhas celulares; Distribuição da sensibilidade das espécies
INTRODUCTION

The pesticide industry is continuously pursuing the discovery of new pesticides acting at novel molecular targets to cope with pathogen resistance and simultaneously being less toxic to humans and to the environment. One such group of novel synthetic organic compounds is the strobilurin chemical group of fungicides. Their discovery was based on the identification, in the wood-rot fungi, *Oudemansiella mucida* and *Strobilurus tenacellus*, of a group of active natural compounds displaying a potent activity against yeasts and filamentous fungi (Sauter et al., 1999). The synthesis of an anti-fungi substance provides this Basidiomycota mushrooms with the ability to defend themselves, allowing them to keep their competitors at a distance and even destroying them (Kettering et al., 2004). However, strobilurin natural compounds were found to be unsuitable as agricultural fungicides because they are unstable under natural sunlight conditions (Bartlett et al., 2001). Independent programs of research using strobilurin natural compounds as chemical models started within ICI (presently part of Syngenta) and BASF Crop Protection Global, and in November 1992 the synthetic analog azoxystrobin was presented at the Brighton Conference (Bartlett et al., 2001). Azoxystrobin was the first patent of the strobilurin compounds which entered primarily in the German market in February 1996 under the trade name of Amistar® (Bartlett et al., 2001; Sauter et al., 1999). Amistar® has two different formulations, a water-dispersible granule containing 500 g kg\(^{-1}\) of azoxystrobin and a suspension concentrate containing 250 g L\(^{-1}\) of the active substance. Suspension concentrate formulations as single active substance products are presently registered under different trade names, e.g., Abound®, Ortiva®, Priori® or Quadris®. However, formulations that
combine active substances can also be found in the market, e.g., Quilt® (azoxystrobin 7.0% + propiconazole 11.7%). All of these related brands are now the world's No. 1 fungicide, registered for use in over 100 countries by Syngenta (Syngenta website in 2015). The strobilurins are considered an outstanding new class of fungicides since they are post-emergence broad-spectrum systemic fungicides, preventing and/or curing foliar diseases caused by the major groups of pathogenic plant fungi - Ascomycota, Deuteromycota, Basidiomycota and Oomycota, and its high success results from its capacity to control combinations of pathogens, which was previously only possible through the mixture of two or more fungicides (Bartlett et al., 2001). According to Syngenta website, commercial products containing azoxystrobin as active substance are now registered for use in over 130 different crops and can control soil-borne and foliar diseases such as rusts, powdery mildew, downy mildew, black rot, scab, anthracnose, white mold, rhizoctonia limb, peg rot, early and late leaf spot, black sigatoka, botrytis, web blotch and rice blast (Bartlett et al., 2001; FAO Meeting, 2008). These products inhibit spore germination and mycelial growth, and also show antisporelant activity. The molecular target of strobilurin-related fungicides is the mitochondrial respiratory complex III (also called cytochrome c oxidoreductase or cytochrome bc1 complex), which is an integral membrane protein complex that couples electron transfer from quinol to cytochrome c1 to proton translocation across the membrane. Strobilurins specifically inhibit mitochondrial respiration by blocking electron transfer between cytochrome b and cytochrome c1, at the ubiquinol oxidizing site (Qo) of the mentioned complex, inhibiting the mechanism by which this complex achieves energy resulting from oxidation-reduction reactions (Bartlett et al., 2002; Hnatova et al., 2003). This inhibition results in cellular oxidative stress triggered by electrons escaping from the mitochondrial respiratory chain and, consequently, this excess of electrons can cause an abnormal generation of reactive oxygen species (ROS) (Kim et al., 2008). A new classification of inhibitors for the cytochrome bc1 complex was
proposed by Esser et al. (2004), being azoxystrobin considered a bc1 inhibitor of the class P, binding to the Qo site; and of the subgroup Pm, having the ability to induce mobile conformation of iron-sulfur protein subunit (ISP). Despite being designed to control fungal pathogens, their general mode of action makes strobilurin-related fungicides as bioactive compounds, toxic not only to target fungi, but also to non-target fungi (e.g., Dijksterhuis et al., 2011), algae (e.g., EFSA, 2010), aquatic plants (e.g., Smyth et al., 1993) and aquatic animals (e.g., Warming et al., 2009), among others. Moreover, Maltby et al. (2009) concluded that the sensitivity of non-target organisms (primary producers, invertebrates and fish) to strobilurin-related fungicides is approximately the same among taxonomic groups.

Each year, the European Pesticide Monitoring Program includes at least one type of different cereal crop for analysis. In 2008, rice crops were monitored and azoxystrobin was one of the ten most frequently found residues on rice (EU Reference Laboratories for Residues of Pesticides, 2012). According to the European Food Safety Authority (EFSA), azoxystrobin was among the most frequently found pesticides in foodstuffs: around 5% of the analysed fruit and vegetable samples contained residues equal or below the tolerance levels (maximum residue limits, MRL’s) (EFSA, 2010). Azoxystrobin is considered to have low acute and chronic toxicity to humans, birds, mammals and bees (Bartlett et al., 2002; EFSA, 2010; US-EPA, 1997). However, despite the absence of critical areas of concern related to non-target species in the azoxystrobin Environmental Risk Assessment (ERA), an exception was made for aquatic organisms, since a data gap was identified after the peer-review of the pesticide risk assessment of EFSA (2010). Furthermore, several authors highlighted that the gap in the current toxicological endpoints of the effects of fungicides in the aquatic environment limits scientific interpretations (Battaglin et al., 2011). In what concerns European pesticide risk assessment, EFSA (2010) concluded that azoxystrobin and its
formulations were considered as very toxic to aquatic organisms, potentially causing long-term adverse effects in the aquatic environment, being considered dangerous. However, azoxystrobin’s more environmental relevant metabolites were described as less toxic (EFSA, 2010). US-EPA (1997) noted that azoxystrobin is toxic to freshwater and marine fish and aquatic invertebrates and issued instructions for keeping it out of lakes, streams, ponds, tidal marshes, or estuaries.

The use of pesticides for crop protection may result in the presence of toxic levels of residues in aquatic matrices due to pesticide mobility through air, during application, or through soil percolation, during irrigation. In the aquatic environment, pesticides might freely dissolve in the water or bind to suspended matter and to sediments, and could be transferred to organisms’ tissues during bioaccumulation processes, likely resulting in adverse consequences to aquatic life and ultimately to human health. However, in the aquatic environment, the occurrence of pesticides is generally documented for specific compartments such as water, and studies on their ultimate fate are missing in the scientific literature (this project, Rodrigues et al., 2013c).

The determination of pesticides in environmental matrices is crucial for the assessment of their environmental risk, as risk is typically characterised in the risk assessment framework as the ratio between exposure concentrations and critical effect concentrations (European Chemicals Bureau, 2003). Seafood consumption is an important route of human exposure to pesticides, thus human health risk assessment also becomes essential (Guo et al., 2007). However, environmental matrices are a chemical analytical challenge because of their high complexity and the presence of low concentrations of the target pesticides. Moreover, a wide spectrum of pesticides with a large range of physico-chemical properties can be found in these matrices.
Biological samples are analytically difficult due to the irreversible adsorption of proteins in the stationary phase of chromatographic techniques, resulting in a loss of column efficiency and an increase in backpressure (Nováková and Vlčková, 2009). Hence, effective sample preparation, as the extraction and clean-up steps prior to chromatographic analysis, which allows high recoveries of the analytes while minimising the presence of matrix interferences, is essential. Moreover, the successful determination of multi-residues in complex matrices requires that the sample preparation procedure be followed by an analytical method that should be sensitive and selective enough to quantify several compounds at trace levels. In addition, currently, the development of modern analytical methodologies only makes sense if the principles of Green Chemistry are complied with, since these principles also encompass laboratory practices.

Several sample preparation techniques are usually used for solid and semi-solid matrices, such as Microwave Assisted Extraction (MAE), Ultrasonic Solvent Extraction (USE), and Liquid-Liquid Extraction (LLE). However, Pressurised Liquid Extraction (PLE) is currently considered an advanced technique since it offers important benefits such as shorter extraction time, decreased solvent consumption and decreased sample handling, when compared to the traditional Soxhlet extraction procedure of biological samples (Giergielewicz-Możajska et al., 2001; Kock-Schulmeyer et al., 2013; Pan et al., 2014; Sosa-Ferrera et al., 2013). Moreover, this technique is in line with the green aspects of sample preparation (Curtylo et al., 2007). For the clean-up step, different solid-phase extraction sorbents such as Florisil® or alumina might be used, which could be placed directly in the extraction cell of the PLE technique (called Selective Pressurized Liquid Extraction (SPLE) technique). For instance, SPLE was successfully applied to the analysis of alkylphenols and bisphenol A in sediments (Salgueiro-González et al., 2014) and mussels (Salgueiro-González et al., 2012). Another alternative for the clean-up step
is the on-line Solid Phase Extraction (on-line SPE) technique, with main advantages, namely high sample throughput, minimal solvent utilization, fast sample preparation and the small sample volume required (5.0-40 mL) (Bones et al., 2006). This technique has been widely applied to environmental analysis, clearly exhibiting better performance than off-line schemes (Pan et al., 2014). For instance, on-line SPE was successfully applied to the analysis of polycyclic aromatic hydrocarbons (PAHs) in sediments by Ericsson and Colmsjö (2002). No other studies based on SPL or on-line SPE for the analysis of pesticides in aquatic matrices were found in the literature.

The present project aims to develop and validate robust analytical methodologies for the determination of atrazine, which was excluded in 2004 as an active substance from Annex I of Directive 91/414/EEC (European Commission, 2004), and other five currently-used pesticides (azoxystrobin, bentazon, λ-cyhalothrin, penoxsulam and terbuthylazine) in marine complex matrices. The SPL technique was used to enable the simultaneous and automatic in-cell clean-up process, which is then followed by multi-residue quantification using on-line SPE clean-up and Ultra Performance Liquid Chromatography-tandem Mass Spectrometry (on-line SPE-UPLC-MS/MS). The methods developed by the present study were applied to real environmental marine samples, such as sediment, the macroalgae *Ulva* spp. (the seaweed Sea lettuce), the aquatic plant *Zostera noltii* and the bivalve *S. plana* (Peppery furrow shell).

Estuarine environments are considered especially rich and diverse ecosystems which provide feed, refuge, and reproduction conditions to aquatic communities, as well as ecosystem services to humans. Even though estuaries are usually located downstream fertile agriculture areas used for intensive agriculture (river valleys), current knowledge of concentration levels of pesticides in estuarine ecosystems is
lacking in scientific literature. Knowledge regarding current environmental concentrations of pesticides are of crucial importance to keep updated data repositories. In Europe, the Marine Strategy Framework Directive (Directive 56/2008/EC) establishes a community action to protect the marine environment. In order to achieve a good environmental status, marine monitoring programmes are required. Moreover, pesticide environmental risks are typically characterised in the risk assessment framework by considering the ratio between exposure concentrations and critical effect concentrations, with exposure assessments based on Predicted Environmental Concentration (PEC) or Measured Environmental Concentration (MEC) values (European Chemicals Bureau, 2003). Even though PEC values are generally determined by the use of model calculations, Pereira et al. (2014) highlight that realistic exposure concentrations are required to validate and eventually calibrate these models. In addition, according to the European Chemicals Bureau, the assessment of the potential impact of pesticides on top predators is based on the accumulation of residues through food chains, which may follow many different paths along different trophic levels, and consequently pose a potential human health risk, since numerous aquatic species are edible and considered of economic interest.

The present work was developed in order to document the occurrence and fate of atrazine and of five current-use pesticides (azoxystrobin, bentazon, λ-cyhalothrin, penoxsulam and terbuthylazine) in water and in complex matrices such as sediment, macroalgae (Ulva spp., G. gracilis and Fucus vesiculosus), aquatic plants (Zostera noltii, Spartina maritima and Scirpus maritimus) and aquatic animals (Nereis diversicolor and S. plana) in the Mondego estuary (Portugal). Atrazine was chosen as it is considered a persistent herbicide (Jablonowski et al., 2010; Smith and Walker, 1989) which was extensively used in the region (in Portugal use was officially allowed until 31st December 2007), and is currently included in the
European 33 priority pollutant list (Annex II, Directive 105/2008/EC), as well as in the Endocrine Disruption Screening Program of the US Environmental Protection Agency (US-EPA) (US-EPA, 2009), whereas the choice of current-use pesticides was based on the technical information of the Regional Direction of Agriculture and Fisheries of the Centre of Portugal. Azoxystrobin is the world’s leading agricultural fungicide (PAN, 2015), bentazon, penoxsulam and terbutylazine are herbicides, and λ-cyhalothrin is an insecticide. In addition, penoxsulam is an herbicide registered for use in aquatic systems. The selected species were chosen as they represent different trophic/functional groups, and the bivalve mollusc S. plana is considered a key species of this estuary (Verdelhos et al., 2005). Moreover, Ulva spp. (Sea lettuce), G. gracilis (Slender wart weed) and S. plana (Peppery furrow shell) are edible species. Pesticide seasonal variations were expected. Hence, the levels of estuary residues before the beginning of and after the production season were studied. A spatial pollutant gradient was foreseen as well. The Mondego estuary is a coastal area of recognised high environmental value integrating the Portuguese Ecological Reserve. The estuary constitutes an important biotope for avifauna (Lopes et al., 2005), particularly for migratory birds (Lopes et al., 2008), and is included in the world list of Important Bird and Biodiversity Areas (BirdLife International PT039). Its wetlands are also considered of international importance, being protected under the Ramsar Convention (Ramsar site No. 1617). In the Mondego estuary, which historically is under the pressure of upstream intense agricultural areas (mainly maize and rice crops) representing about 12,300 ha (Lower Mondego), the occurrence of pesticide residues has not been subject to an overall assessment since 2001-2003 (Almeida et al., 2007). Among the eight Portuguese estuaries studied by Almeida and collaborators, the Mondego estuary showed, at the time, the highest levels of pesticides, as water samples presented substantial contents of molinate (0.8-38.6 μg L\(^{-1}\)) and chlorfenviphos (1.9-3.0 μg L\(^{-1}\)), and in lower concentrations, oxadiazon (<0.1 ng L\(^{-1}\)) and chlorophenols such as
2,4,6-trichlorophenol (TCP, 3.0-7.0 μg L⁻¹), 2,3,4,5-tetrachlorophenol (TeCP, 4.0-5.0 μg L⁻¹) and pentachlorophenol (1.0-12.7 μg L⁻¹). Concerning sediments, samples also showed high levels of molinate (8.0-12.5 ng g⁻¹), bisphenol A (0.1-2.8 ng g⁻¹), TCP (0.2-0.3 μg g⁻¹), TeCP (0.2-0.3 μg g⁻¹), dibutyltin (3.5-9.0 ng g⁻¹) and tributyltin (1.9-4.7 ng g⁻¹).

The pesticides selected for the present study include thiadiazines (bentazon), triazines (atrazine) and chlorotriazines (terbuthylazine), as well as strobilurins (azoxystrobin), pyretroids (λ-cyhalothrin) and triazolopyrimidines (penoxsulam). Further than the rate and frequency of pesticide application, the nature of the sediment, climate conditions, the physico-chemical properties of each pesticide, among others, also influence their fate in the aquatic environment. Table 1 summarises some of the physico-chemical properties of the selected pesticides. Briefly, in water, bentazon and penoxsulam are highly soluble and atrazine is moderately soluble, whereas azoxystrobin and terbuthylazine have low solubility, and λ-cyhalothrin is considered hydrophobic. In water, azoxystrobin, bentazon, λ-cyhalothrin and penoxsulam are stable to hydrolysis at neutral pH, yet atrazine and terbuthylazine may degrade via this chemical reaction. Concerning the tendency for pesticides to be adsorbed by sediments, which may be described by the organic carbon-water partitioning coefficient (Koc), among the studied pesticides, λ-cyhalothrin presents the highest value (Log Koc = 5.5). Moreover, the pesticide bioaccumulation potential can be measured by the octanol-water coefficient (Kow), and, in general, chemicals are considered lipophilic if Log Kow >3, and have the potential to be bioaccumulated if Log Kow >4. Hence, bentazon and therbuthylazine are considered lipophilic, but only λ-cyhalothrin has the potential to be accumulated by organisms. Regarding aquatic systems, the Bioconcentration Factor (BCF) is also a metric to categorise the bioconcentration potential of chemicals. For example, under European regulation concerning the Registration, Evaluation, Authorization
and Restriction of Chemicals (REACH), a BCF >2,000 L Kg\(^{-1}\) indicates bioaccumulative potential, and a BCF >5,000 L Kg\(^{-1}\) very bioaccumulative potential (REACH Regulation, 2007).

Current awareness regarding the potential adverse health consequences associated with pesticide occurrence in aquatic systems is increasing, and thus several worldwide regulations determined pesticide target values for drinking water and aquatic habitats. For instance, the Regulation SI No. 278 is the legislation that sets out parametric values for pesticides in drinking water within the European Union, being 0.1 μg L\(^{-1}\) the maximum contamination level applied to each pesticide individually, and 0.5 μg L\(^{-1}\) the sum of all individual pesticides detected and quantified in the course of a monitoring procedure (European Commission, 2007b).

The World Health Organization also has specific pesticide guideline values for drinking water quality (WHO, 2011). Aquatic life benchmarks for freshwater species are also target values established by the US-EPA for the protection of aquatic organisms. Moreover, atrazine has a specific maximum concentration allowed for European estuarine waters (Annex I, Directive 105/2008/EC) and the European Food Safety Authority set RAC values for some pesticides in order to protect aquatic organisms. Table 2 summarises the target values reported for the selected pesticides.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Physico-chemical and environmental properties of the studied pesticides based on TOXNET database information.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alachlor</td>
</tr>
<tr>
<td>CAS No.</td>
<td>1912-24-9</td>
</tr>
<tr>
<td>Molecular weight (g mol(^{-1}))</td>
<td>215.684</td>
</tr>
<tr>
<td>Water solubility (mg L(^{-1}))</td>
<td>28 (20°C)</td>
</tr>
<tr>
<td>pH 5</td>
<td>244 (pH 4, 25°C)</td>
</tr>
<tr>
<td>pH 7</td>
<td>-</td>
</tr>
<tr>
<td>pH 9</td>
<td>-</td>
</tr>
<tr>
<td>Koc</td>
<td>64-546 (sediments)</td>
</tr>
<tr>
<td>Kow</td>
<td>Log Kow: 2.61</td>
</tr>
<tr>
<td>BCF in fish (L Kg(^{-1}))</td>
<td>&lt;0.27-100</td>
</tr>
</tbody>
</table>

*Koc, carbon adsorption coefficient; Kow, n-octanol: water partitioning coefficient; BCF, bioconcentration factor
In the present work, edible species were collected to provide some indications regarding the extent of the bioaccumulation of pesticide residues within an estuarine ecosystem since estuarine species are of the upmost economical importance and are the most exploited natural resources, and thus may represent a major pathway for human contamination. However, to our knowledge, there are currently no guideline or regulation limits specifically for concentrations of the selected pesticides in seaweeds and shellfish.

It is known that toxicity testing is widely conducted as a way to determine the potential human health and environmental impairment of pollutants worldwide, and animal models are required to allow the classification of chemicals according to their intrinsic toxicity. However, animal welfare concerns have, for a long time, shown the intent to reduce the number of sacrificed fish worldwide in aquatic toxicological research, and the use of lower organisms as surrogates for vertebrates is one of the alternatives. Recently, there is increasing evidence of the pivotal role of the crab *C. maenas* in assessing the impact of pollutants, especially in the toxicological assessment of coastal and transitional waters (this project, Rodrigues and Pardal, 2014). In the present project, an extensive review of literature was conducted to find relevant research on the use of *C. maenas* as an experimental test organism in ecotoxicology, in order to find if it can be used as a model organism in ecotoxicology and environmental quality assessments.
Commonly known as European Green crab, the macroinvertebrate *C. maenas*, a marine portunid brachyuran crustacean, has been widely used in aquatic ecotoxicology research over the last 45 years (from Portmann (1968) to present). Interest in this species lies in the fact that *C. maenas* is one of the best-studied estuarine organisms, with a well known biology and ecology (e.g., Behrens Yamada, 2001). Additionally, the isolation of 25% of the total gene number of *C. maenas* by Towle and Smith (2006) has become particularly relevant to support gene-based studies. In ecotoxicity testing, *C. maenas* offers ethical advantages when compared to fish. This crab also fulfils many of the criteria of the protocol for selection of sentinel species and collection of specimens (EROCIPS, 2006). For instance, it is common and widespread, it is very easy to identify, it reproduces sexually and is sexually dimorphic (adult male and female are readily identifiable on the basis of their morphology). Moreover, it holds a central position in the marine food chain, making the linkage between primary producers and other invertebrates and top predators. Finally, it is commercially important in some regions, very easy to catch from wild populations and also easy to maintain in the laboratory. This crab is one of the species listed as a test organism in the American Society for Testing and Materials (ASTM) standard guide for conducting acute toxicity tests on test materials (ASTM E729, 2002). In the European context, the study of the effects of environmental pollutants at ecosystem level is a requirement of the Water Framework Directive (WFD, Directive 60/2000/EU, 2000), and macroinvertebrate benthic communities are considered key components for the assessment of benthic integrity in view of the mentioned Directive. Furthermore, *C. maenas* is one of the species selected for the ECOMAN programme, a multi-biomarker approach used for the discrimination between contaminated and clean sites and applied in ecosystem management (Galloway et al., 2006).
Natural habitats of C. maenas are considered as high risk habitats, for they are environmentally sensitive areas, i.e., this epibenthic organism lives in close association with estuarine/marine sediments where toxic levels of a large variety of chemicals from urban, industrial, agricultural and maritime activities (pollutants) may accumulate. Pollutants particularly adversely affect estuaries, as these are always close to urban and industrial centres. Moreover, estuarine upstream fertile river floodplains are usually used for intensive agriculture. Estuarine waters are also used for transport and recreation, and for the development of port and boating facilities. Accordingly, pollutants as pharmaceutical drugs, endocrine disruptors, PAHs, polychlorinated biphenyls (PCBs), pesticides and metals enter estuaries through storm drains, discharges from sewage and industrial wastewater treatment plants, discharges, leaching and runoff processes from agricultural areas, and atmospheric deposition. Despite awareness and efforts to limit discharges into coastal and transitional waters, e.g., through the OSPAR Convention or the WFD, these inputs continue to impair aquatic ecosystems, thus strengthening an ever growing need to select adequate marine animal experimental models and develop ecotoxicology tools so as to understand how aquatic organisms interact with pollutants in their environment and determine potential environmental and human health hazards. Ultimately, the main goal of aquatic ecotoxicology is to attain better regulatory decision-making and risk reduction.

According to the Intergovernmental Panel on Climate Change (IPCC), the Earth’s climate is changing due to carbon emissions driven by human fossil fuel combustion and deforestation (IPCC, 2013). Thus, in the context of the global warming phenomenon, aquatic organisms are expected to deal with the rising mean temperatures and frequency of temperature extremes involved in climate-driven events. Variations in temperature are of particular importance to aquatic ectotherms
(e.g., *C. maenas*), as their body temperature is in constant equilibrium with the surrounding environment. An experimental study was therefore designed as part of this project in order to gain ecologically relevant data and provide novel scientific knowledge of biochemical (mitochondrial SOD and GST activities) and physiological (mitochondrial function) responses of *C. maenas* to azoxystrobin (30 µg L⁻¹) in the context of climate change projections. Concerning temperature, *C. maenas* adults are eurythermic, with preferences ranging between 3°C and 26°C (Grosholz and Ruiz, 2002). Survival is low when water temperature remains below 3°C for two consecutive months or more (Breen and Metaxas, 2009), and feed ceases somewhere between 2°C and 7°C (Ropes, 1968). Moreover, it appears that at least 10°C are necessary for them to moult, and that below that temperature their activity is drastically reduced (Berrill, 1982; Eriksson and Edlund, 1977). Its Critical Thermal Maximum is around 35°C, with seasonal variations (Cuculescu et al., 1998).

Concerning pesticide residues, they enter the crab from water, sediment, or food; via the gill or stomach, and accumulate in the hepatopancreas via haemolymph (Brouwer and Lee, 2007). The crustacean hepatopancreas has many of the functions associated with the vertebrate liver, pancreas, and small intestine, as food absorption, transport, secretion of digestive enzymes, and storage of lipids, glycogen and a number of minerals (Brouwer and Lee, 2007; Felgenhauer, 1992). Moreover, it is known that the crabs’ hepatopancreas is considered a suitable organ in determining organisms’ responses to toxic levels of chemicals since it is the main metabolic site and the target detoxification organ (this project, Rodrigues and Pardal, 2014). In his review concerning oxidative stress in aquatic organisms, Lushchak (2011) reports an enhancement of oxygen consumption, and then a possible increase of ROS production as a result of temperature rise. Moreover, azoxystrobin may also be able to produce ROS, since the generation of hydrogen peroxide (H₂O₂) by the pesticide myxothiazol, which has the same mode of action
as azoxystrobin, was demonstrated by Starkov and Fiskum (2001) in rat heart and brain mitochondria. Usually, a balance exists between the production of ROS and antioxidant processes, but organism’s unbalance situations may occur with potential consequences on membrane damage and enzyme inactivation (Livingstone, 2001). *C. maenas*’s antioxidant enzymes include SOD, catalase (CAT) and glutathione peroxidase (GPx), and GST, which is an enzyme of *phase II* detoxification metabolism, which can also directly detoxify free radicals (this project, Rodrigues and Pardal, 2014). On the other hand, the successful use of pesticide toxicity assessments using mammalian liver mitochondrial preparations in order to measure oxidative and phosphorylative capacities is well documented by Moreno and Madeira (1990 and 1991), Moreno et al. (2007), and Palmeira et al. (1994, 1995) in what concerns parathion, DDT, carbaryl, 2,4-D and dinoseb, and paraquat, respectively. However, to our knowledge, the mentioned approach was only applied to aquatic invertebrates using the Mediterranean mussel *Mytilus galloprovincialis* by Nesci et al. (2011). Mitochondria as an experimental model are being recognised as a key factor in many areas of biomedical science since plays, among others, a well-known crucial contribution in organisms’ oxidative phosphorylation and metabolism (Smith et al., 2012). Nevertheless, its contribution to ecotoxicology studies has not been widely used.

The development of alternative assays, such as cell-based assays, has also become an important topic of interest by this project. However, the replacement of fish lethal testing by cell-based assays can only be effective if high-level correlations between both assays are found and similar absolute sensitivities are attained. A good correlation between *in vivo* fish results and *in vitro* fish cytotoxicity data, specifically regarding relative sensitivity, has already been confirmed (Kramer et al., 2009). However, when correspondence is considered in absolute terms, fish cells
have so far proved to be less sensitive than whole fish (Castaño et al., 2003; Kramer et al., 2009). Thus, it has been stated that fish cell-based assays include a certain risk of false negative results (Castaño et al., 2003). Therefore, scientific knowledge concerning comparative studies, in vivo/in vitro, with the aim to find sensitive cell models, fish or mammal derived, are crucial. Also, in the universe of scientific literature, there is a great amount of data concerning cytotoxicity results, even though most are related to drugs for human use and only a few are ecotoxicologically relevant. Thus, in order to accelerate the development of new alternative methods, testing environmentally relevant hazardous substances is also essential.

Regarding azoxystrobin, data on aquatic toxicity are scarce (this project, Rodrigues et al., 2013c), with only four fish azoxystrobin LC$_{50}$ values available: Oncorhynchus mykiss: LC$_{50,96h}$ = 470 (400-5,800) µg L$^{-1}$ (US-EPA, 1997), Ctenopharyngodon idella larvae (<10 days): LC$_{50,48h}$ = 549 (419-771) µg L$^{-1}$ (Liu et al., 2013), Lepomis macrochirus: LC$_{50,96h}$ = 1,100 (900-1,700) µg L$^{-1}$ (EPA, 1997), and the euryhaline species Cyprinodon variegatus: LC$_{50,96h}$ = 670 (560-800) µg L$^{-1}$ (US-EPA, 2012a). In addition, azoxystrobin toxicity has been only tested using adrenocortical H295r and hepatocellular HepG2 carcinoma cell lines (Prutner et al., 2013; Rudzoka et al., 2009). The chosen endpoints were oestrone production by H295r and cytochrome P450 1A induction by HepG2. Nevertheless, none of these studies determined IC$_{50}$, i.e., the concentration of azoxystrobin required to achieve 50% in vitro cell inhibition. Therefore, this project discloses the environmental significance of cell-based assays as an alternative to fish toxicity tests by comparing the response of in vitro cell-based assays using four mammalian and two fish cell lines with the juvenile gilthead seabream in vivo lethal test in order to contribute to the development of testing methods alternative to the use of laboratory animals.
The use of single-species toxicity data is well established as a key component for the determination of pesticide aquatic effects and risk assessment, as well as for water quality guideline derivation, and is accepted by worldwide environment authorities (OSPAR Convention, 2000; REACH regulation, 2006). Even though assays are conducted under laboratory conditions, results are generally accepted as a conservative estimate of the potential effects of pollutants in the field, thus providing useful data to assess individual sensitivities. However, the complex organisation of aquatic systems demands higher relevant approaches in order to protect communities or even ecosystems, such as the SSD concept. This approach allows to determine the maximum exposure concentration at which an ecosystem is protected (i.e., protective of ecosystem structure and function), and subsequently derive water quality guidelines for the protection of aquatic life. For instance, the PNEC value for the aquatic compartment used to determine the risk quotient in environmental risk assessment could be derived by SSD curves (European Chemicals Bureau, 2003). The Assessment Factor (AF) method is another alternative to establish PNEC values. The SSD approach assembles single-species toxicity data in order to predict hazardous concentrations (HC₅) affecting a certain percentage (x) of species in a community. The most conservative form of this approach uses the lower 95% tolerance limit of the estimated percentage to ensure that the specified level of protection is achieved. Hose and Van den Brink (2004) confirmed this concept of species protection by comparing laboratory-based SSD curves with both local mesocosm experiments and field monitoring data. SSD curves are constructed by fitting a cumulative distribution function to a plot of species toxicity data against rank-assigned percentiles (Wheeler et al., 2002). The greater the number of species tested, the lower the uncertainty of the risk assessment attributable to interspecies differences in sensitivity. According to Newman et al. (2000), sample size producing HC₅ (hazardous concentration for 5% of species) estimates with minimal variance should range from 15 to 55. However,
the Society of Environmental Toxicology and Chemistry (SETAC) guidance document on higher tier risk assessment for pesticides (“HARAP-Report”) states that, in general, a dataset of acute single-species assays on eight organisms representing primary producers, crustaceans and fish can be used to describe the distribution of sensitivities of aquatic organisms (SETAC, 1999).

The azoxystrobin active ingredient is presently registered under different trade names, such as Amistar®, Ortiva®, among others. The latter is a mixture of declared hazardous components which are reported in its Safety Data Sheet: 22.9% w/w of azoxystrobin and 10-20% w/w of propane-1,2-diol (Syngenta, 2010). The present study used AF and SSD approaches to estimate six PNEC values for the fungicide azoxystrobin, which were applied to freshwater and marine toxicity datasets for azoxystrobin, as well as to a marine toxicity dataset for Ortiva®. A selected PNEC value was then validated and recommended as the PNEC value for azoxystrobin in the aquatic environmental compartment. This target value is a fundamental key tool to allow ERA as risks are typically characterized by considering the ratio between exposure concentrations and critical effect concentrations.

Environmental risk assessment is a scientific step-wise procedure. After azoxystrobin aquatic exposure and effect assessment, the next step is its risk characterization, which is the likelihood of adverse effects in the aquatic environmental compartment due to exposure concentrations. To a certain extent, this procedure allows for a retrospective reality check of the prospective registration procedure for pesticides under Regulation by the European Commission (European Commission, 2009). Concerning the risk characterization of azoxystrobin for the aquatic environmental compartment, under a first approach it could be determined by the results of the present project, since it is calculated as the ratio MEC/PNEC.
(RQ). The obtained RQ is then compared against a value of one, and if RQ > 1, a risk for the environment cannot be excluded and further assessment is recommended.
Chapter I – A review


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5.664 IF_{2013} (21 citations (all), web of knowledge), Q1_{2013} Environmental sciences
Occurrence, Fate and Effects of Azoxystrobin in Aquatic Ecosystems

The present Chapter had the intent to conduct an extensive literature review to find relevant research on the occurrence, fate and effects of azoxystrobin in aquatic ecosystems in order to identify strengths and gaps in the scientific database. Analytical procedures and existing legislation and regulations were also assessed.

Abstract

The use of pesticides for crop protection may result in the presence of toxic levels of residues in aquatic systems. In the aquatic environment, residues might freely dissolve in the water or bind to suspended matter and to the sediments, and might also be transferred to the organisms’ tissues during bioaccumulation processes, resulting in adverse consequences to non-target species, and ultimately to humans. One such group of synthetic organic pesticides widely used worldwide to combat pathogenic fungi affecting plants is the strobilurin chemical group. Whereas they are designed to control fungal pathogens, their general modes of action are not specific to fungi. Consequently, they can be potentially toxic to a wide range of non-target organisms. After an extensive literature review to find relevant research on the occurrence, fate and effects of azoxystrobin, the first patent of the strobilurin compounds, in aquatic ecosystems, it was possible to identify strengths and gaps in the scientific database. Data gathered in the review revealed, at that time, that analytical reference standards for the most relevant environmental metabolites of azoxystrobin are needed. Validated confirmatory methods for complex matrices like sediment and aquatic organisms’ tissues are very limited. Important knowledge of
base-line values of azoxystrobin and its metabolites in natural tropical and marine ecosystems is lacking. Moreover, some environmental concentrations of azoxystrobin found in the review are above the Regulatory Acceptable Concentration (RAC) in what concerns risk to aquatic invertebrates and the No Observed Ecologically Adverse Effect Concentration (NOEAEC) reported for freshwater communities. The review also showed that there are very few data on azoxystrobin toxicity to different aquatic organisms, especially in what concerns marine organisms. Besides, toxicity studies mostly address azoxystrobin and usually neglect the more relevant environmental metabolites. Further work is also required in what concerns effects of exposure to multi-stressors, e.g., pesticide mixtures. Even though the octanol-water partition coefficient (Kow) for azoxystrobin and R234886, the main metabolite of azoxystrobin in water, are below 3, the bioconcentration factor and the bioaccumulation potential for azoxystrobin are absent in the literature. Moreover, no single study on bioaccumulation and biomagnification processes was found in the present review.

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**Azoxystrobin physico-chemical characterization**

Azoxystrobin is the ISO approved name for methyl \((E)\)-2-\{2 [6-(2-cyanophenoxy)pyrimidin-4-yl]oxy\} phenyl)-3-methoxyacrylate (IUPAC) and for methyl \((E)\)-2-\{[6-(2-cyanophenoxy)-4-pyrimidinyl]oxy\}-\(\alpha\)-(methoxymethylene) benzeneacetate (9CI) (CA). This carboxylic acid methyl ester has the molecular formula of \(\text{C}_{22}\text{H}_{17}\text{N}_{3}\text{O}_{5}\) and the molecular mass of 403.4 g mol\(^{-1}\). Its CIPAC and CAS registry numbers are 571 and 131860-33-8, respectively. The US-EPA chemical code is 128810. Azoxystrobin retains the methyl \(\beta\)-methoxyacrylate group of the naturally-occurring strobiurins (Bartlett et al., 2001), which characterizes its structure (Fig. 1). The molecule of azoxystrobin also has a large hydrophobic moiety of three aromatic rings: a
cyanophenyl ring, a pyrimidinyl ring, and a phenylacrylate ring. However, it is considered a moderately polar compound (Smalling and Kuivila, 2008). At 20°C, the solubility of azoxystrobin in water is 6.7 mg L\(^{-1}\) (pH 5.2 and 7.0) and 5.9 mg L\(^{-1}\) (pH 9.2), while in organic solvents it is 0.057 g L\(^{-1}\) for hexane, 1.4 g L\(^{-1}\) for octan-1-ol, 20 g L\(^{-1}\) for methanol, 55 g L\(^{-1}\) for toluene, 86 g L\(^{-1}\) for acetone, 130 g L\(^{-1}\) for ethyl acetate, 340 g L\(^{-1}\) for acetonitrile and 400 g L\(^{-1}\) for dichloromethane (European Commission, 1998). Azoxystrobin may have a stereoisomer but its active substance is the \(E\) form.

Since azoxystrobin has several functional groups, it is considered as having complex metabolic pathways with the formation of a large number of metabolites (Roberts and Hutson, 1999). For instance, Parra et al. (2012a) presented the synthesis and the complete spectroscopic characterization of breakdown compounds widely recognised as major metabolites of azoxystrobin, such as the so-called acid and enol derivatives and the azoxystrobin Z-isomer. Moreover, using computer predictions and high-resolution mass spectrometry, Kern et al. (2009) screened the potential transformation products of azoxystrobin in natural aquatic environments and an ester hydrolysis product (also called azoxystrobin acid or R234886) was concluded to be the main metabolite found. Identically, Singh et al. (2010) detected R234886 as the main product of \(^{14}\text{C}\)-azoxystrobin in water. Thus, R234886, also \((E)-2\text{-}[2\text{-}[6\text{-}[2\text{-cyano-phenoxy]} \text{ pyrimidin-4-} \text{loxy]} \text{ phenyl}]\text{-3-} \text{methoxyacrylic acid, can be formed from azoxystrobin, either by hydrolysis of the}
ester group or by oxidative de-alkylation, and is considered moderate soluble (57 mg L\(^{-1}\) at 20\(^\circ\)C) by IUPAC (2012). Moreover, EFSA (2010) also defined 4-(2-cyanophenoxy)-6-hydroxypyrimidine (R401553), a product of the cleavage of the ether linkage between the phenyacrylate ring and the pyrimidinyl ring, and 2-[6-(2-cyanophenoxy) pyrimidin-4-yl]benzoic acid (R402173) as environmental relevant metabolites of azoxystrobin. Boudina et al. (2007) studied the aqueous photochemical behaviour of azoxystrobin and results suggested that, in water, phototransformation proceeds via multiple, parallel reaction pathways including photo-isomerisation, photo-hydrolysis of the methyl ester and of the nitrile group, cleavage of the acrylate double bond, photohydrolytic ether cleavage between the aromatic ring resulting in phenol, and oxidative cleavage of the acrylate double bond. This study also concluded that azoxystrobin in aqueous solution absorbs light at wavelengths higher than 290 nm and can therefore be photodegraded in the environment. Moreover, (Z)-methyl 2-[6-(2-cyanophenoxy) pyrimidin-4-yl]phenyl]-3-methoxyacrylic acid (also called Z-isomer or R230310) was identified as the main metabolite of photochemical transformation, also under ultraviolet (UV) irradiation, being photo-isomerisation a very fast reaction occurring immediately upon irradiation.

**Environmental behaviour, fate and occurrence**

After field application, residues of pesticides can remain in the air, soil or water, being runoff and/or leaching processes the main transport pathways from soil to the surrounding water bodies. Physico-chemical properties of residues affect its behaviour and consequently its introduction and distribution into environmental compartments. The vapor pressure, at 25\(^\circ\)C, of azoxystrobin is 1.1×10\(^{-7}\) mPa (IUPAC, 2012). This low value indicates that after released into the air (e.g., through drift during spraying), azoxystrobin will remain mainly in the particulate phase, which
can be removed from the atmosphere either by wet or dry deposition. The low volatilization rate (Henry’s Law Constant, at 20°C) of $7.3 \times 10^{-14}$ atm m$^3$ mol$^{-1}$ for azoxystrobin also corroborates the latter (IUPAC, 2012), as well as the estimated atmospheric half-life shorter than 2 days presented by EFSA (2010) and the nearly 3% of volatilization losses presented by Singh et al. (2010) after 130 days of $^{14}$C-azoxystrobin incubation.

When a pesticide reaches the soil, it interacts with organic and mineral constituents and undergoes biological and chemical transformations (Bending et al., 2006). The occurrence of microbes adapted to use pesticides as energy sources makes microbial degradation one of the first possible routes for pesticide loss (Bending et al., 2003). In the soil, under aerobic conditions, azoxystrobin degrades with a median dissipation time value, DT$_{50}$, between 56 and 279 days, depending on the chemical and microbial properties of the soil, and no significant degradation was observed in sterile treatments (EFSA, 2010; FAO Meeting, 2008), suggesting that the aerobic degradation is mainly due to microbial activity. Studies also showed differences in degradation rates under anaerobic and aerobic conditions, with half-life values of 107.47 and 62.69 days in aerobic and anaerobic soils, respectively, indicating that azoxystrobin is more persistent in aerobic than in anaerobic soils (Ghosh and Singh, 2009a). In the soil, the major residue is the parent compound azoxystrobin and the only significant metabolite is R234886, being this metabolite moderately to highly persistent (DT$_{50}$ of 18 to 44 d) (EFSA, 2010; FAO Meeting, 2008; Ghosh and Singh, 2009a). During labelled azoxystrobin experiments in the soil, it was reported that formations of unextractable residues, considered a sink, account for 6.2 to 24.5% of the applied radioactivity after 120 days (EFSA, 2010).

Although azoxystrobin is not an ionic pesticide, Bending et al. (2006) found that azoxystrobin sorption onto the soil was related to pH, with sorption decreasing as pH declined. Furthermore, they found a strong correlation between azoxystrobin
sorption and DT$_{25}$, with the degradation rate decreasing as sorption increased. They concluded that, in the soil, pH can induce differences in azoxystrobin bioavailability and has a role in controlling its degradation rate. In the upper layers of soil, photodegradation occurs with a half-life of 11 days (US-EPA, 1997), and Ghosh and Singh (2009a) concluded that both sunlight and UV light degrade azoxystrobin, being the degradation at a faster rate under UV light. Azoxystrobin exhibits relatively low binding affinities (distribution coefficient, Kd 1.5-4.0 L kg$^{-1}$) on coarse textured soils (e.g., loamy sand and sand) and higher binding affinities (Kd 5.0-23 L kg$^{-1}$) on finer textured soils (US-EPA, 1997). The distribution coefficient describes the distribution of a compound between the solid and the liquid phases, indicating higher Kd values a stronger adsorption to the soil matrix. Bending et al. (2007) calculated the Kd of 13.9 L kg$^{-1}$ (mean value) one day after azoxystrobin application to sandy loam soils containing 73% sand, 12% silt, 14% clay and 1.2-1.8% of total organic carbon. Ghosh and Singh (2009b) studied the leaching behaviour of azoxystrobin and the metabolite R234886 in packed and intact soil columns under different irrigation regimes. Results indicate that azoxystrobin is fairly immobile in sandy loam soil, but azoxystrobin acid is quite mobile. The metabolite R234886 is considered also by EFSA (2010) as having very high to high mobility with a Kd of 0.5 to 14 L kg$^{-1}$. Simulation models also suggested that azoxystrobin presents a risk to water quality as a result of runoff processes from treated fields (Deb et al., 2010).

In what concerns water matrices, azoxystrobin is considered stable to hydrolysis and has low solubility (6.7 mg L$^{-1}$, at 20°C) (EFSA, 2010; IUPAC, 2012; US-EPA, 1997). Singh et al. (2010) studied the behaviour of $^{14}$C-azoxystrobin in water at different pH values (4, 7 and 9) and found that the formation of the major metabolite of azoxystrobin, R234886, was faster and in larger quantities under alkaline (pH 9) conditions. This study also concluded that azoxystrobin is hydrolytically fairly stable at a pH between 4 and 9, appearing to degrade at a slightly faster rate at alkaline
pH. The rate of azoxystrobin decomposition in aquatic environment induced by light or other radiant energy is considered moderately fast by IUPAC (2012) and has an aqueous photolysis DT$_{50}$ at pH 7 between 8.7 and 13.9 days (European Commission, 1998). In an outdoor pond study, azoxystrobin dissipated from the water column with a calculated DT$_{50}$ of about 13 days and in the adjacent sediment it was continuously increasing in the first three weeks of the study (EFSA, 2010), indicating that once azoxystrobin reaches water, it will be quickly adsorbed onto sediment and subsequently degraded. Azoxystrobin dissipation rates from water with half-life values of 18 days and 15 to 25 days were also determined by Zafar et al. (2012) and Gustafsson et al. (2010), respectively. In order to compare three pesticide risk indicator model outputs under realistic Norway pesticide management regimes, Stenrød et al. (2008) classified the mean leaching risk of azoxystrobin as medium/high and medium in what concerns marine sand and moraine deposits, and marine silt clay deposits, respectively. In laboratory conditions, in incubations with aerobic natural sediment, azoxystrobin exhibited high persistence (SFO DT$_{50}$ of 180 to 234 d), forming the major metabolite R234886 (EFSA, 2010). The Log Kow of 2.5 (at 20°C without pH dependence) presented by EFSA (2010) and IUPAC (2012) characterizes the low liposolubility of azoxystrobin, indicating that it would not potentially generate bioaccumulation and biomagnification processes (European Commission, 2011). The higher the Kow, the higher the probability of a residue binding to organic matter in water and sediments, or entering living tissues. Kern et al. (2009), using an increment method, estimated a Log Kow of 2.2 for the metabolite R234886. To our knowledge, and after an extensive literature review, no data of the bioconcentration factor (BCF) or the bioaccumulation potential of azoxystrobin was available. Also, no single work was found regarding bioaccumulation studies on aquatic organisms. Meanwhile, the on-line TOXNET database presents an azoxystrobin BCF of 21 L Kg$^{-1}$ for fish and Lazartigues et al.
(2013) estimated the azoxystrobin biomagnification factor (BMF) for two freshwater fish species, being $3.4 \times 10^{-4}$ and $5.6 \times 10^{-4}$ for perch and carp, respectively.

The background knowledge of natural exposures of azoxystrobin and R234886 residues found worldwide is presented in Tables 3 and 4 for water and sediment samples, respectively. Furthermore, in what concerns R234886, EFSA (2010) concluded that the potential for groundwater exposure to this metabolite is predicted to be high over a wide range of geoclimatic conditions, since the concentration of this metabolite was estimated to be above $10 \mu g L^{-1}$ over a range of FOCUS (organization co-sponsored by the EU and industry) groundwater scenarios, and it was identified as a critical area of concern.

### Table 3 Dissolved azoxystrobin and R234886 concentration ($\mu g \text{ L}^{-1}$) in natural water samples.

<table>
<thead>
<tr>
<th>Country</th>
<th>Location</th>
<th>Aquatic system</th>
<th>No. samples</th>
<th>Collecting period</th>
<th>Residue</th>
<th>Detection frequency (%)</th>
<th>Mean concentration ($\mu g \text{ L}^{-1}$)</th>
<th>Maximum concentration ($\mu g \text{ L}^{-1}$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>US</td>
<td>13 States</td>
<td>Streams (29)</td>
<td>103</td>
<td>2005/2006</td>
<td>Azoxystrobin</td>
<td>45.0</td>
<td>0.16</td>
<td>1.13</td>
<td>Batsagin et al. (2011)</td>
</tr>
<tr>
<td></td>
<td>Maine, Idaho, Wisconsin</td>
<td>Streams, ponds (12)</td>
<td>80</td>
<td>2009</td>
<td>Azoxystrobin</td>
<td>58.0</td>
<td>0.03</td>
<td>0.06</td>
<td>Reilly et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>Maine, Idaho, Wisconsin</td>
<td>Groundwater (13)</td>
<td>12</td>
<td>2009</td>
<td>Azoxystrobin</td>
<td>17.0</td>
<td>0.0009</td>
<td>0.0009</td>
<td>Reilly et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>Colorado, Montana and Wyoming high plains national parks</td>
<td>Lakes, creeks (15)</td>
<td>26</td>
<td>summer 2009</td>
<td>Azoxystrobin</td>
<td>3.8</td>
<td>0.06</td>
<td>0.06</td>
<td>Kestela (2011)</td>
</tr>
<tr>
<td>Brazil</td>
<td>Nepal, Sri Lanka</td>
<td>Surface, groundwater</td>
<td>54</td>
<td>2009/2010</td>
<td>Azoxystrobin</td>
<td>9.3</td>
<td>-</td>
<td>0.16</td>
<td>Smidting et al. (2012)</td>
</tr>
<tr>
<td>Denmark</td>
<td>Experimental field sites</td>
<td>Groundwater</td>
<td>26</td>
<td>October 2009</td>
<td>Azoxystrobin</td>
<td>11.5</td>
<td>0.15</td>
<td>0.19</td>
<td>Fihlo et al. (2010)</td>
</tr>
<tr>
<td>Germany</td>
<td>Lower Saxony, Braunschweig</td>
<td>Experimental field sites</td>
<td>450</td>
<td>2004-2009</td>
<td>Azoxystrobin</td>
<td>24.4</td>
<td>0.05</td>
<td>1.40</td>
<td>Jorgensen et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>Lower Saxony, Braunschweig</td>
<td>Experimental field sites</td>
<td>473</td>
<td>2004-2009</td>
<td>R234886</td>
<td>53.5</td>
<td>0.15</td>
<td>2.10</td>
<td>Jorgensen et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>Lower Saxony, Braunschweig</td>
<td>Experimental field sites</td>
<td>1173</td>
<td>2004-2009</td>
<td>Azoxystrobin</td>
<td>&lt;1.0</td>
<td>0.01</td>
<td>0.01</td>
<td>Jorgensen et al. (2012)</td>
</tr>
</tbody>
</table>

### Table 4 Azoxystrobin concentration ($\mu g \text{ Kg}^{-1}$) in natural bed sediment samples.

<table>
<thead>
<tr>
<th>Country</th>
<th>Location</th>
<th>Aquatic system</th>
<th>No. samples</th>
<th>Collecting period</th>
<th>Residue</th>
<th>Detection frequency (%)</th>
<th>Mean concentration ($\mu g \text{ Kg}^{-1}$)</th>
<th>Maximum concentration ($\mu g \text{ Kg}^{-1}$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>US</td>
<td>Alabama, California, Georgia, Washington</td>
<td>Creeks (7)</td>
<td>7</td>
<td>2005-2007</td>
<td>Azoxystrobin</td>
<td>42.0</td>
<td>3.60&lt;sup&gt;0&lt;/sup&gt;</td>
<td>12.60</td>
<td>Smalling and Kunst (2006)</td>
</tr>
<tr>
<td></td>
<td>7 States</td>
<td>Amphibian habitats, ponds</td>
<td>42</td>
<td>2009/2010</td>
<td>Azoxystrobin</td>
<td>11.9</td>
<td>-</td>
<td>12.60</td>
<td>Smalling et al. (2012)</td>
</tr>
<tr>
<td>Portugal</td>
<td>Mondego estuary</td>
<td>Intertidal areas</td>
<td>8</td>
<td>March, August 2014</td>
<td>Azoxystrobin</td>
<td>11.9</td>
<td>3.92</td>
<td>3.92</td>
<td>Rodrigues et al. (unpublished)</td>
</tr>
<tr>
<td></td>
<td>Melbourne</td>
<td>urban and peri-urban wetlands (24)</td>
<td>24</td>
<td>April 2010</td>
<td>Azoxystrobin</td>
<td>4</td>
<td>7</td>
<td>-</td>
<td>Allison et al. (2015)</td>
</tr>
</tbody>
</table>

<sup>a) concentration below the detection limit and estimated by authors</sup>
Analytical methodology

Sample preparation

There is substantial variation in the published information for collection and preservation of samples for azoxystrobin analysis, but all agree that the use of new amber glass, teflon or aluminum extrusion containers is preferred. Water samples should be filtered through a 0.45 μm membrane to remove particulate matter (Filho et al., 2010; Kern et al., 2009; Polati et al., 2006), but several authors also used a 0.20 μm membrane (Liess and von der Ohe, 2005) or a 0.70 μm membrane (Bony et al., 2008; Rabiet et al., 2010; Reilly et al., 2012; Smalling et al., 2012). Since residues of azoxystrobin were stable at ≤ −18°C for up to two years in matrices with high water-, high acid-, and high fat content as well as in dry matrices, in order to prevent any matrix degradation before analysis, EFSA (2010) recommend that samples should be stored frozen for a maximum of 10 months. If frozen, samples should be thawed out at +4°C. Furthermore, a refrigerated (0-5°C) storage stability study was performed in water samples by Hsu et al. (2010) and results showed no significant degradation of azoxystrobin, azoxystrobin acid or Z-isomer for 28 days.

An effective sample preparation allowing high recoveries of the analyte while minimizing the presence of interferences is needed. Depending on the sample matrices, azoxystrobin literature extraction methods are based on different extraction methodologies. In what concerns water samples, extraction methods are mainly based on solid phase extraction (Battaglin et al., 2011; Bony et al., 2008; Boudina et al., 2007; Jørgensen et al., 2012; Kern et al., 2009; Lazartigues et al., 2011; Liess and von der Ohe, 2005; Polati et al., 2006; Rabiet et al., 2010; Reilly et al., 2012; Smalling et al., 2012). For multi-residue analysis of water and groundwater samples, Filho et al. (2010) successfully developed a Solid Phase Micro Extraction method based on the direct immersion mode (DI-SPME) by exposing 2.4 cm length of an 85 μm polyacrilate fiber to the sample solution. The
Microwave-Assisted Solvent Extraction (MASE) is usually applied for sediment samples (Smalling and Kuivila, 2008), and more recently, pressurize liquid extraction methods using accelerated solvent extractor equipments were also used (Smalling et al., 2012). Moreover, also for sediment samples and for organisms' tissues, a single solid-liquid extraction method was successfully applied by Lazartigues et al. (2011). Following extraction, a clean-up step is normally performed in sediment samples, as well as in organisms' tissues samples, prior to instrumental analysis. Concerning water samples, a pre-concentration procedure with a concentration factor of 1,000 should be performed before extraction (Bony et al., 2008; Polati et al., 2006; Rabiet et al., 2010). Filho et al. (2010) showed that pH had no effect on the extraction of water samples.

Screening methods

The majority of the instrumental screening methods were developed for food stuff matrices due to food safety concerns. In groundwater, drinking water and surface water, residues of azoxystrobin can be monitored by a Gas Chromatography-mass Selective Detector (GC-MSD) (EFSA, 2010) or by a Gas Chromatograph-Electron Capture Detector (GC-ECD) (Berenzen et al., 2005; Liess and von der Ohe, 2005). In what concerns sediment samples, a multi-residue method was successfully achieved by da Silva et al. (2011), using comprehensive two dimensional gas chromatography with micro-electron capture detection. Residues of azoxystrobin in animal matrices can be performed by Gas Chromatography-Nitrogen Phosphorous selective Detection (GC-NPD) (EFSA, 2010). Advanced screening antibody-based technologies were also developed to indicate the presence of azoxystrobin or its metabolites in a sample. In this type of immunoassays, no false negative results are allowed. They were developed based on the principle that natural binding characteristics of antibodies make them ideal as potential markers and identifiers for
the presence of their corresponding specific antigen. Furzer et al. (2006) described the production of polyclonal antibodies against azoxystrobin by using the acidic form of the molecule for direct conjugation, and Parra et al. (2012b) developed an enzyme-linked immunosorbent assay (ELISA) optimised in the conjugate-coated indirect competitive format (i-cELISA). This immunoassay was based on four functionalised bioconjugates of azoxystrobin previously described in Parra et al. (2011). They generated a panel of monoclonal antibodies with subnanomolar affinity for azoxystrobin or its metabolites which could be applicable to monitor food and environmental samples.

Confirmatory methods

Adequate confirmatory analytical methods, all using mass spectrometry, are presently available for the determination of azoxystrobin and its metabolites. Since it is considered a non-volatile compound (IUPAC, 2012), liquid chromatography methods are suitable and successfully applied. However, for multi-residue analysis, gas chromatography-mass spectrometry is usually used. For identification purposes, standards can be diluted with methanol (Filho et al., 2010; Hsu et al., 2010), acetone (Rabiet et al., 2010) or acetonitrile (Polati et al., 2006). In what concerns water samples, several mass spectrometry methods were developed, namely: High Pressure Liquid Chromatography-Ultra Violet detection (240 nm detection wavelength) tandem Mass Spectrometry (HPLC-UV-MS/MS/MS) by Polati et al. (2006), Liquid Chromatography-Mass Spectrometry with a Triple Quadrupole (LC-MS/MS-Q3) by Zafar et al. (2012), Liquid Chromatography Mass Spectrometry with Atmospheric Pressure Ionization with a positive source polarity (LC-MS/MS-APCI) by Hsu et al. (2010), Liquid Chromatography-tandem Mass Spectrometry using a Linear Trap Quadrupole (LC-MS/MS-LTQ) by Kern et al. (2009), and Liquid Chromatography Electrospray Ionization-tandem Mass Spectrometry (LC-MS/MS-
ESI) by Bony et al. (2008), Boudina et al. (2007), Lazartigues et al. (2011), Rabiet et al. (2010) and Villeneuve et al. (2011). The latter method was also used by Bony et al. (2008) in biofilm samples and in organisms' tissues by Lazartigues et al. (2011). A LC-MS/MS-APCI method was used by Boudina et al. (2007) in their studies of azoxystrobin aquatic photodegradation. For multi-residue analysis of water samples, Gas Chromatography-Mass Spectrometry with Selected Ion Monitoring (GC-MS, SIM) was successfully applied by Battaglin et al. (2011) and Filho et al. (2010), whereas Gas Chromatography-Mass Spectrometry with Electron Ionization (GC-MS, EI) was successfully applied by Reilly et al. (2012) and Smalling et al. (2012). The latter method was also used by Smalling et al. (2012) for multi-residue analysis of sediment samples, operated in selective ion monitoring mode. Gas chromatography with negative chemical ionisation coupled to a quadruple mass spectrometer was also used by Liess and von der Ohe (2005) for the same type of matrix.

**Ecotoxicity to aquatic organisms**

Freshwater organisms

The toxicity of azoxystrobin to freshwater organisms gathered from single-species tests for aquatic and sediment dwelling organisms is presented in Table 5. Nevertheless, other toxicity studies were performed exposing aquatic organisms to azoxystrobin, being results presented below. For instance, the effect of the imidazole fungicide prochloraz in a binary mixture with azoxystrobin was studied by Cedergreen et al. (2006) using the algae *Pseudokirchneriella subcapitata* and the aquatic plant *Lemna minor*. However, no synergy was observed in the mixture tested against both species. Moreover, Warming et al. (2009) evaluated the chronic physiological effects of azoxystrobin on three clones of *Daphnia magna* (clones Gammelmosen, Herlev Gadekær and Langedam) originating from different Danish
lakes and results showed that through respiration measurements and life-table experiments, sublethal stress was shown to exist at exposure to the environmental concentration of 0.026 μg L⁻¹. Furthermore, the effect on egg-carrying *D. magna* of sublethal concentrations of azoxystrobin (500; 1,000 and 2,000 μg L⁻¹) on the activity of several physiological parameters (heart, filtering limbs, mandibles and focal spine) during 24 hours was studied by Friberg-Jensen et al. (2010). Results point out that the focal spine of egg-carrying *D. magna* was not affected by azoxystrobin whereas the activity of all other response parameters decreased by exposure to 500 μg L⁻¹ (EC₄₋₂₄ₜₖ) of azoxystrobin. In addition, it is known that aquatic fungi and bacteria are responsible for the decomposition and conversion of riparian plant litter into more palatable food resources for macroinvertebrates, being the latter the second major group of organisms that make the conversion of leaf litter into secondary production and fine particulate organic matter possible. With the intent to study the impact of pesticides on leaf litter decomposition in agricultural streams, Rasmussen et al. (2012) surveyed pesticide contamination (a total of 20 pesticides, including azoxystrobin) and rates of leaf litter decomposition in Danish streams. The toxicity of the measured pesticide concentrations for microbial and macroinvertebrates organisms was quantified using Toxic Units (TU), relating the concentration of a pesticide in a natural aquatic system with its toxicity, based on EC₅₀ values. Results showed that microbial litter decomposition was reduced by a factor of two to four in agricultural streams compared to forested streams, and that the rate of microbial litter decomposition responded strongly to pesticide toxicity, being −1.92 the Log₉ maximum TU attributed to azoxystrobin. In what concerns macroinvertebrates, azoxystrobin was found to be the second most contributor to high values of Log₉ maximum TU (−2.77). In the latter case, determinations were based on LC₅₀,₄₈ₜₖ of *D. magna* (259 μg L⁻¹). In order to study effects of pesticides on invertebrate communities, Liess and von der Ohe (2005) used the same procedure to compare the toxicity present during runoff events on Germany streams.
Azoxystrobin was one of the four pesticides contributing the most to the TU (between −2.30 and −1.37), being classified by authors as one of the most toxic substances and reported that it had lethal to sublethal effects on the invertebrate communities investigated. The effect of multiple stressors, e.g., mixture of several pesticides and their degradation products, on aquatic organisms can also be assessed. For example, the joint effect of prochloraz together with azoxystrobin on *D. magna* was studied by Cedergreen et al. (2006). Immobility after 48 hours was recorded. Results highlighted the strong synergy effect found on *D. magna* with sums of TU for the 50:50% effect mixture (ΣTU₅₀:₅₀) as low as 0.46, indicating that only 46% of the fungicide azoxystrobin was needed to immobilize 50% of *D. magna* in the presence of prochloraz, compared to the azoxystrobin acting alone. In order to study the potential of the downstream drift of amphipoda *Gammarus pulex* triggered by a sublethal concentration of azoxystrobin (nominal concentration of 20 μg L⁻¹), Beketov and Liess (2008) referred that results did not show a drift initiating effect for azoxystrobin and concluded that drift-initiating action by some pesticides might result in a significant change of the structure of a lotic community.

Amphibians typically have both terrestrial and aquatic life stages and, therefore, can also be susceptible to the effects of pesticides. With the intent to compare the effect of the active substances azoxystrobin and propiconazole, and the corresponding commercial formulation Quilt®, Hooser et al. (2012) performed lethal essays (96 h) with *Bufo cognatus* tadpoles. Results suggested that neither the active substances nor the formulation Quilt® appear to pose a risk toward amphibian larvae under normal field use conditions. Mortality averaging was less than 60% at the highest concentrations (1,200 μg L⁻¹ azoxystrobin + 2,000 μg L⁻¹ propiconazole). Moreover, there were no differences between the effects of the active substances and the commercial formulation. In order to study the toxicity of six pesticides, Johansson et al. (2006) performed lethal (72 h) and sublethal (from fertilization to metamorphosis)
tests with common frog (Rana temporaria) tadpoles. Azoxystrobin was one of the three most lethal pesticides in the acute exposure and had also negative effects on the growth of the tadpoles. However, negative effects were only observable at the highest concentration, considered as non-environmental (0.5 mg L\(^{-1}\)). Moreover, no significant effect was observed in the sublethal treatments with azoxystrobin.

Table 5 Ecotoxicological endpoints for freshwater organisms reported after single-species tests (short and long-term). AZX = azoxystrobin technical substance.

<table>
<thead>
<tr>
<th>Group (Dinoflagellate)</th>
<th>Species and life cycle stage</th>
<th>Residue</th>
<th>Endpoint and exposure time</th>
<th>Concentration (mg L(^{-1}))</th>
<th>Confidence limits (95%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. magna</td>
<td>AZX</td>
<td>Growth</td>
<td>EC(_{50}), 72h</td>
<td>106</td>
<td>92-121</td>
<td>US EPA (2012a)</td>
</tr>
<tr>
<td>D. magna</td>
<td>AZX</td>
<td>Growth</td>
<td>EC(_{10}),14d</td>
<td>120</td>
<td>110-130</td>
<td>US EPA (2011c)</td>
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Table 5 Ecotoxicological endpoints for freshwater organisms reported after single-species tests (short and long-term). AZX = azoxystrobin technical substance.

Higher ecological relevance studies were also performed using azoxystrobin, being the results presented below. For instance, Rimet and Bouchez (2011) developed a diatom-based tool with the intent to assess pesticide contamination in rivers. This
study used a lotic mesocosm approach (63-75 d) in several experiments (acute and chronic), during which the effects of environmental concentrations of the herbicide diuron (0.03-13.03 μg L⁻¹) and the fungicides azoxystrobin (0.60-7.22 μg L⁻¹) and tebuconazole (0.39-6.60 μg L⁻¹) were tested on benthic diatom metrics, namely: ecological guilds, life-forms and cell size. Results showed that pesticide contamination was the second most important parameter related to structuring diatom communities after colonisation time, and had a more significant impact on the composition of ecological guilds than on species composition. Results also showed that the metrics benthic/planktonic, colonial, pedunculate and pioneer did not display any significant trends, whereas abundances of motile guild, low-profile guild and mucous tubule diatoms increased in contaminated channels, and high-profile diatoms showed the opposite trend. Moreover, the effect of chemical and physical factors on periphyton structure, diversity and functioning were investigated in an outdoor mesocosm in a 67-d experiment by Villeneuve et al. (2011). Natural benthic microbial communities, mostly composed of diatoms, cyanobacteria and chlorophyceae, were subjected to a mixture of 2.7 μg L⁻¹ (mean value) of the herbicide diuron and 1.2 μg L⁻¹ (mean value) of azoxystrobin, under two different hydraulic regimes (turbulent with high variations and laminar with low variations). Results showed that this pesticide mixture modified the structure, diversity and functional efficiency of the algal community, being this community denser and less productive than that in the reference. Pesticides also displayed specific species diversity with some species of diatom only identified in the context of pesticide contamination. In what concerns the bacterial community, higher densities were observed in the presence of pesticides. They also concluded that communities which developed in turbulent mesocosms were more diversified. However, the highest biodiversity found did not increase the ability of these biofilms to tolerate pesticides. Furthermore, Zafar et al. (2012) developed a set of experiments with the intention of evaluating the effect of different time-varying exposure patterns of
Azoxystrobin on freshwater microcosm communities, using the commercial formulation Amistar®. Several phytoplankton, macrophytes, zooplankton and macroinvertebrate species were used and four treatment regimes were applied, namely: a continuous application treatment of 10 a.i. µg L\(^{-1}\) for 42 days; a continuous application treatment of 33 a.i. µg L\(^{-1}\) for 42 days; a single application treatment of 33 a.i. µg L\(^{-1}\); and four applications, each achieving a peak of 16 a.i. µg L\(^{-1}\) with a time interval of 10 days. Results showed that the largest adverse effects were reported for zooplankton taxa belonging to copepoda and cladocera and that azoxystrobin only slightly affected some species of macroinvertebrate, phytoplankton and macrophyte assemblages. This work also showed that, for long-term effect studies, the time-weighted average regime is a more adequate predictor for most zooplankton species than the peak concentration. Data gathered from the continuous application treatment of 10 a.i. µg L\(^{-1}\) for 42 days allowed setting 10 µg L\(^{-1}\) as NOEAEC, being the concentration at or below which no long-lasting adverse effects were observed in the microcosm study. Moreover, in fungicides, the relationship between HC\(_1\) and HC\(_5\) values (hazardous concentration to 1% or 5% of the tested taxa in a species sensitivity distribution approach) and threshold values from micro and mesocosm experiments was analysed by Maltby et al. (2009). Authors concluded that HC\(_5\) values were not always protective against acute effects of fungicides, but derived lower-limit HC\(_5\) (LLHC\(_5\)) or the median HC\(_5\) divided by an assessment factor of 3 were always protective of adverse ecological effects on aquatic primary producers, invertebrates and litter breakdown in semi-field studies. The median HC\(_5\) reported in this study for azoxystrobin was 42 µg L\(^{-1}\). In what concerns fish, the genotoxic effect in erythrocytes of chronic (0.5-1.0 µg L\(^{-1}\)) and acute (7.0 µg L\(^{-1}\)) concentrations of azoxystrobin was assessed by the measurement of DNA damage using the Comet assay by Bony et al. (2008) using early life stages of brown trout, *Salmo trutta fario*. Despite being qualitative, results
highlighted that azoxystrobin can represent a genotoxic threat to freshwater fish from contaminated watershed rivers.

Marine organisms

The azoxystrobin toxicity to marine organisms reported after single-species tests is presented in Table 6. However, other studies showing azoxystrobin toxicity to marine organisms were also provided. For instance, a study at the subcellular level using the commercial formulation Amistar® was developed by Olsvik et al. (2010). Results found that CAT, IGFBP1 and MAPK1 gene transcripts were significantly up-regulated in the liver of juvenile Atlantic salmon-smolt (Salmo salar), and concerning muscle tissue, five genes showed a significantly altered expression - catalase, IGFBP1, transferrin, TNFR (up-regulated) and CYP1A (down-regulated). They concluded that azoxystrobin affects mitochondrial respiration by interfering with mechanisms controlling cell growth and proliferation in fish. These mechanisms include oxidative stress and apoptosis and triggered an adaptive protective response through the IGFBP1 gene.

A higher ecological relevance study (microcosm approach) regarding the effects on the community structure and function in brackish waters was developed by Gustafsson et al. (2010) in the Baltic Sea with natural plankton communities and sediment. Results showed that all tested concentrations, nominal concentrations of 3.0, 7.5, 15 and 60 µg L\(^{-1}\), clearly altered the structure of the zooplankton community by reducing the abundance of copepod nauplii and increasing the abundance of the rotifers Synchaeta spp. Moreover, the composition of the
phytoplankton community was also altered. This study also concluded that azoxystrobin is toxic to brackish water copepods at considerably lower concentrations (≤3 μg L⁻¹) than previously reported for single-species tests performed with freshwater crustaceans.

To our knowledge, and after an extensive literature review, no data was found regarding the toxicity of the most relevant metabolites of azoxystrobin to marine organisms. Moreover, knowledge on how strobilurins may interact with other contaminants, often found together with this residue in the aquatic environment, and its joint effects on marine organisms were also lacking.

Legislation and regulation

Before any pesticide can be used commercially, several tests are conducted for a preliminary risk characterization. These determine whether it has any potential to cause adverse effects on humans and wildlife, including endangered species and other non-target organisms, or potential to contaminate surface waters and groundwater from leaching, runoff and spray drift. Therefore, the European environmental risk assessment of plant protection products relates to the individual active substances, and depending on the outcome of the EU risk assessment, an active substance may be included in a positive list - Annex I to Council Directive 414/91/EEC, i.e., can be authorized at the level of the member states during a predetermined period (Directive 414/91/EEC, 1991). In accordance with the provisions of Article 6(2) of the mentioned directive, the authorities received on 15th September 1995 an application from Zeneca Pesticides for the inclusion of the active substance azoxystrobin in the mentioned annex of the Directive. Azoxystrobin gained Annex I inclusion as a new active substance under the EU Directive in July 1998 and in 2007 a renewal process extended the inclusion until 31st December 2011 (European Commission, 2007a). In 2010, the Commission Directive

Considering consumer risk assessment, the Acceptable Daily Intake (ADI) and the Acceptable Operator Exposure Level (AOEL) to azoxystrobin are set at 0.2 mg kg\(^{-1}\) bw day\(^{-1}\), applying an assessment factor of 100 (EFSA, 2010). No Acute Reference Dose (ARfD) is allocated to azoxystrobin (EFSA, 2010; FAO Meeting, 2008). In 2012, the European MRLs for azoxystrobin in foodstuffs ranged between 0.01 mg kg\(^{-1}\) (e.g., milk, curd) and 70 mg kg\(^{-1}\) (e.g., parsley, rosemary). Azoxystrobin’s MRLs, were firstly set in Annex II of Commission Regulation 396/2005/EC, and several posterior amends were included over the years, the last one in Commission Regulation 270/2012/EU (European Commission, 2005, 2012). The parametric limit for a single pesticide in both drinking water and groundwater is 0.1 μg L\(^{-1}\) (Directive 83/98/EC, 1998; Directive 118/2006/EC 2006).

In order to determine the risk to aquatic invertebrates, EFSA (2010) expressed concern since insufficient data were available which could fulfill regulatory requirements. However, EFSA (2010) considered that it was possible to determine a RAC, and defined it as 3.3 μg a.i. L\(^{-1}\).

US-EPA Benchmarks are chemical concentrations, specific to either water or sediment, above which there is the possibility of harm or risk to humans or animals in the environment. The Office of Pesticide Programs (OPP) in EPA provides annually Benchmark values for individual pesticides on its website to aid in the
assessment of potential risk to fish and other aquatic life. For azoxystrobin, the following aquatic life Benchmarks for freshwater species are available in the US-EPA (2012b): 235 μg L\(^{-1}\) for acute toxicity in fish, 147 μg L\(^{-1}\) for chronic toxicity in fish, 130 μg L\(^{-1}\) for acute toxicity in invertebrates, 44 μg L\(^{-1}\) for chronic toxicity in invertebrates, 49 μg L\(^{-1}\) for acute toxicity of aquatic non vascular plants and 3,400 μg L\(^{-1}\) for acute toxicity of aquatic vascular plants.

Health Canada's Pest Management Regulatory Agency (PMRA) signed in 1998 a Memorandum of Understanding (MOU) with Environment Canada to facilitate the exchange of information and advice regarding pest control products. Under the MOU, Environment Canada carries out environmental research and monitoring and provides the results to PMRA to assess risks associated with pesticides. Thus, in April 2003 the PMRA identified a total of 18 pesticides to Fisheries and Oceans Canada as being of national concern, and azoxystrobin was one of the listed compounds. However, it was classified as low priority at that time (Verrin et al., 2004).

Concluding remarks

The intensive use of pesticides for crop protection may lead to the contamination of air, soil, surface and groundwater, increasing concern for the risk of impacts in the surrounding aquatic ecosystems. Effects in non-target species may result in ecosystem unbalance and food-web disruption, which may affect edible species and, ultimately, human health, making food web biomagnification studies a critical component of aquatic ecological risk assessment. The development of such risk assessment is a scientifically demanding process which requires the production of a vast range of data. Data gathered in the present review indicate that azoxystrobin is a strongly sorbed pesticide in soil matrices, which is more persistent in aerobic soils than in anaerobic ones and, unlike what happens in the water, in the soil, its
degradation is pH dependent. The major metabolite and degradation compound of azoxystrerin aerobic soils, anaerobic soils, water and water-sediment systems is R234886 (EFSA, 2010; Ghosh and Singh, 2009a; Singh et al., 2010), being diffused by leaching and runoff processes (Ghosh and Singh, 2009b; Jørgensen et al., 2012). In what concerns the parent compound azoxystrerin, Deb et al. (2010) and Jørgensen et al. (2012) considered that azoxystrerin can also occur in runoff processes, being, as well as R234886, a potential environmental pollutant which can degrade surface water quality. Although azoxystrerin-related commercial products are relatively stable with regard to photodegradation under natural sunlight conditions, Boudina et al. (2007) considered this an important process of dissipation in the environment, being its main metabolite the azoxystrerin Z-isomer (R230310).

Outcomes from the present review reveal that analytical reference standards for the most relevant environmental metabolites of azoxystrerin are needed. In what concerns analytical methodologies, newer screening methods like ELISA and monoclonal antibody for detection purposes with high specificity and sensitivity have overcome the limitations of classical methods. However, validated confirmatory methods of azoxystrerin and its metabolites in complex matrices still remain a challenge, being found in the present review only in the works of Bony et al. (2008) for biofilm samples, Smalling and Kuivila (2008) and Smalling et al. (2012) for sediment samples, and Lazartigues et al. (2011) for sediment and fish muscle samples.

Although the environmental occurrence of pesticides may be more frequent in areas of manufacturing and application, the present review showed that azoxystrerin can be found in remote high elevated lakes. The water concentrations of azoxystrerin found in natural environments and displayed in the present review were, in general, one or more orders of magnitude less than toxicity estimates in aquatic life Benchmarks for freshwater species. However, the worst-case exposure
concentration reported in the present review was triple of the NOEAEC determined by Zafar et al. (2012) for freshwater communities and ten times higher than the RAC in what concerns risk to aquatic invertebrates. Thus, azoxystrobin has the potential to occur in concentrations which are above their exposure thresholds. One of the objectives of the European WFD is to reach a “good status” for European rivers by 2015 (Directive 60/2000/EC, 2000). The implementation of the WFD implies the intensification of the monitoring of pesticide residues, as well as of other chemical compounds, the identification of the causes of degradation, and the employment of corrective actions to obtain a good chemical and biological status. For this purpose, Member States have to ascertain a comprehensive monitoring strategy to establish exposure levels in surface waters and to evaluate the water quality improvement linked to various management programs. Data gathered in the present review highlights that to achieve the WFD purposes, important knowledge of base-line values in natural systems must still be generated, especially in what concerns marine environments.

The present review also reveals that there are very few data on azoxystrobin toxicity to different aquatic organisms, and it must be stressed that the vast majority of the available information, which comes directly from the pesticide industry, is present both in the registration requirements for azoxystrobin and in the regulatory documents. These studies mostly address azoxystrobin and usually neglect the more relevant environmental metabolites. As regards azoxystrobin toxicity, the present review shows that the physiological impact on aquatic organisms differs from one species to another and even from one clone to another. The freshwater diatom *N. pelliculosa* was found to be the most sensitive species, with an EbC\(_{50,120h}\) of 14 µg L\(^{-1}\) (EFSA, 2010). Available model ecosystem studies suggested that zooplankton seems to be a sensitive group to azoxystrobin, being the naupliar stages of copepods the most sensitive (Gustafsson et al., 2010; Zafar et al., 2012).
Studies on the possible long-term effects due to the continuous exposure to sublethal concentrations of azoxystrobin are also very limited in the scientific literature. Even though organisms may not die, they can have their functional activities compromised under sublethal concentrations, representing a possible disruption in the function of the ecosystems. Recent molecular approaches of functional genomics, such as transcriptomics, proteomics and metabolomics, are considered as promising techniques to increase detection sensitivity of organisms exposed to sublethal concentrations of chemicals (ecotoxicogenomics). However, only the study of Allen et al. (2004) was found in the current scientific literature relating a metabolic footprint method and cells of the yeast *S. cerevisiae* exposed to azoxystrobin in order to assess its mode of action. Furthermore, marine species are of the most important and exploited natural resources and may, therefore, represent the major pathway for human contamination through bioaccumulation and biomagnification processes. Nevertheless, a gap was also identified in what concerns ecotoxicology studies performed with these organisms. There is also a data gap in what concerns bioaccumulation and biomagnification knowledge, being bioaccumulation kinetics studies considered very important to estimate the potential for environmental harm by Connell et al. (1999). However, as already mentioned, the TOXNET database currently presents an azoxystrobin BCF of 21 (L Kg$^{-1}$) for fish and Lazartigues et al. (2013) estimated the azoxystrobin BMF for two freshwater fish species (*Perca fluviatilis* and *Cyprinus carpio*), being 3.4x10$^{-4}$ and 5.6x10$^{-4}$ for perch and carp, respectively. With the exception of US-EPA Benchmarks, the NOEAEC reported for freshwater communities and the RAC defined for aquatic invertebrates, no other azoxystrobin concentration thresholds, which protect aquatic life, were found in the present review. Further work is also required in what concerns effects of exposure to multi-stressors (chemical and physical), since only the works of Cedergreen et al. (2006), Rimet and Bouchez (2011) and Villeneuve et al. (2011) regarding pesticide mixtures were found. To conclude, the present work identifies
knowledge gaps which don't allow uncertainties to be reduced in what concerns azoxystrobin ecological risk assessment.

Finally, during this review, a lack was also identified regarding data on the occurrence, behaviour, fate and effects of azoxystrobin in tropical aquatic environments. Generating such specific information is essential in order to improve pesticide risk assessment and management decisions in those regions. Daam and Van den Brink (2010) highlighted the differences between temperate and tropical freshwater ecosystems as regards ecological risk assessment tools of pesticides. Authors recommend that more field studies on pesticide fate in the enclosed and surrounding waterways in tropical farms are needed. This study emphasized that despite natural climate and ecosystem sensitivity differences, an intensive agricultural practice in tropical countries leads to a higher input of pesticides and spread of contamination over watersheds, with consequences in the potential toxicity to aquatic organisms.
Chapter II – A field case study

Rodrigues ET, MA Pardal, N Salgueiro-González, S Muniategui-Lorenzo, MF Alpendurada (submitted to Analytica Chimica Acta) A single-step pesticide extraction and clean-up multi-residue analytical method by selective pressurised liquid extraction followed by on-line solid phase extraction and ultra performance liquid chromatography-tandem mass spectrometry for complex matrices

4.513 IF\textsubscript{2014}, Q1\textsubscript{2014} Analytical chemistry

Rodrigues ET, MF Alpendurada, F Ramos, MA Pardal (to be submitted to Water Research) Seasonal and spatial occurrence and fate of pesticides in the Mondego estuary (Portugal).

5.528 IF\textsubscript{2014}, Q1\textsubscript{2014} Water science and technology
A SINGLE-STEP PESTICIDE EXTRACTION AND CLEAN-UP MULTI-RESIDUE ANALYTICAL METHOD BY SELECTIVE PRESSURISED LIQUID EXTRACTION FOLLOWED BY ON-LINE SOLID PHASE EXTRACTION AND ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY FOR COMPLEX MATRICES

This study aims to present the development and validation of robust analytical methodologies for the chemical determination of atrazine, which was excluded as an active substance from Annex I of Directive 91/414/EEC (European Commission, 2004), and other five currently-used pesticides (azoxystrobin, bentazon, λ-cyhalothrin, penoxsulam and terbuthylazine) in complex matrices.

Abstract

To successfully determine pesticide multi-residue (atrazine, azoxystrobin, bentazon, λ-cyhalothrin, penoxsulam and terbuthylazine) levels in complex marine matrices such as sediment, macrophytes and aquatic animals, adequate analytical methodologies were developed and validated by this project. The established methodology applies Selective Pressurised Liquid Extraction (SPLE) followed by on-line Solid Phase Extraction and Ultra Performance Liquid Chromatography-tandem Mass Spectrometry (on-line SPE-UPLC-MS/MS). Parameters such as solvent used, temperature and number of cycles for SPLE have been optimised, as well as a simultaneous and automatic in-cell clean-up. This cutting-edge research methodology uses a small amount of sample (in the order of milligrams), is time saving and reduces the use of organic solvents in compliance with Green Chemistry principles. The analytical features were adequate for all compounds in all studied
matrices: recoveries varied between 49 and 119% and repeatability was lower than 22%. Uncertainty assessment of measurement was estimated on the basis of an in-house validation according to the EURACHEM/CITAC Guide. The quantification limits of these methods ranged between 1.2 (atrazine) and 17 (λ-cyhalothrin) ng g\(^{-1}\) dw for sediment, 6.8 (terbuthylazine) and 17 (penoxsulam) ng g\(^{-1}\) dw for macrophytes, and 11 (azoxystrobin) and 290 (λ-cyhalothrin) ng g\(^{-1}\) dw for animals. Therefore, this innovative analytical methodology could also be applied to food safety analyses.

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**Materials and Methods**

Analytical standards and solutions

The following analytical standards were used in the present study: atrazine 99.1% (Riedel-de Haen), azoxystrobin 99.4% (Fluka), bentazon 99.7% (Riedel-de Haen), λ-cyhalothrin 97.8% (Fluka), penoxsulam 96.9% (Fluka) and terbuthylazine 98.8% (Fluka). Standard individual and mixture stock solutions were prepared in methanol (Carlo Erba, HPLC-Plus) and stored at 4 ± 2ºC.

For the extraction procedures, methanol, acetonitrile and acetone were purchased from Sigma-Aldrich. The sorbents used in the clean-up process such as neutral alumina, basic alumina, silica gel, Florisil® and primary-secondary amine (PSA) were obtained from Supelco. For the liquid chromatographic mobile phase preparation, the methanol Chromasolv® HPLC-grade and ammonium acetate used came from Sigma-Aldrich. Ultrapure water was purified in a Direct MilliQ water system.
Site description and sampling procedures

Samples were collected in 2014 in the Mondego estuary, which is a shallow warm-temperate intertidal system located on the west coast of Portugal. Due to the absence of certified reference materials of sediments, macrophytes (algae and aquatic plants) and aquatic animals for method optimisation and validation, fresh material was collected during low tide in the most downstream area of intertidal flats exposed during low tide (40°07.815′N; 8°49.692′W). The collection of material was carried out a few days after the end of the rainy season and when the work for the productive season in the Lower Mondego was not yet started, which, in 2014, corresponded to the first days of March. During collection, samples were stored in aluminium extrusion containers and transported to the laboratory in 12V car refrigerators. Sediment samples were frozen immediately upon arrival at the laboratory. Macrophytes (*Ulva* spp. and *Z. noltii*) were rinsed briefly in running tap water and gently scrubbed with paper towels to remove most surface microbial and epiphytic organisms, and then frozen. Aquatic animals were represented in the method optimisation and validation process by aquatic worms (*N. diversicolor*) and bivalves (*S. plana*). Both organisms are predominantly surface deposit-feeders of sedimentary organic matter. Therefore, after collection, worms were maintained in aerated recirculating aquatic systems composed of glass tanks (25 × 20 × 9.5 cm) and appropriate life support systems in a 20°C temperature-controlled room and under a natural light regime for gut clearing purposes. Each tank was filled with 2.0 L of reconstituted water at a salinity of 17 (tropic marin salt, Tropical Marine Centre) according to ASTM E729 (2002) guideline. After 72 hours the organisms were dried with paper towels and frozen. Concerning bivalves, *S. plana* were purchased alive in a local food shop and were maintained for depuration purposes under the same conditions as worms. After 96 hours the organisms were dissected and frozen. The frozen macrophytes and animals were freeze-dried (UniEquip Unicryo MC-4L) and
then grinded (Ika T18 basic or Ika MF10 basic), and after homogeneition, were saved in new amber glass vials (Supelco 27004 and 27182). A mixture (50:50%) of *Ulva* spp. and *Z. noltii*, and a mixture (50:50%) of *N. diversicolor* and *S. plana* homogenised tissues was used for macrophyte’s and organism’s method optimisation and validation, respectively.

The material for confirmatory analyses (samples of sediment, *Ulva* spp., *Z. noltii* and *S. plana*) were collected during low tide in a Mondego estuary’s upstream sampling station (40°07.308’N; 8°50.515’W) in August 2014, which represents the highest probability of the presence of pesticide residues in the estuary. Sediment samples were randomly collected (top 5 cm) in triplicate within an area of 100 m² using a stainless steel core (5 cm ∅), and frozen immediately upon arrival at the laboratory. Before being frozen, all the collected macrophytes were rinsed briefly in running tap water and gently scrubbed with paper towels. The procedure for *S. plana* samples was similar to the above described. After depuration, *S. plana* were measured using an electronic digital caliper (VWR 1819-0012) and separated in five size classes (0-1, 1-2, 2-3, 3-4 and >4 cm) corresponding, according to Verdelhos et al. (2005), to 0+, 1+, 2+, 3+ and 4+ year old individuals, respectively. The most abundant size class in the study area was 3+, and, therefore, only this size class was dissected, dried with paper towels, and frozen. The procedure for frozen samples was similar to the one described for method optimisation and validation.

**SPLE method optimisation**

**Sediment**

Multi-residue extraction of sediment samples was performed using the Thermo Scientific™ Dionex™ ASE™ 350 Accelerated Solvent Extractor system. Since SPLE efficiency can be affected by parameters such as extraction solvent,
temperature, or sorbent, among others (e.g., Camino-Sánchez et al., 2011), an optimisation study was performed prior to analysis. Therefore, 1.0 g of homogenised and sieved (1.0 mm mesh) sediment was mixed with 0.25 g of diatomaceous earth (dispersant agent, Thermo 062819) until homogenisation. Then, the sediment was spiked with a small amount of the standard mixture stock solution at a concentration level of 50 ng g\(^{-1}\) dw (dry weight) for all compounds and placed into a 5.0 mL-extraction cell of stainless steel which was sealed at both ends with cellulose filters (Thermo 068093). The extraction was carried out for two hours in order to achieve realistic interactions between the analytes and the matrix. Methanol (MeOH), acetonitrile, and a mixture of acetone:MeOH (50:50 v/v) were tested in triplicate for solvent optimisation. SPLE conditions were: pressure, 1,500 psi; temperature, 60ºC; static time, 3 min.; number of cycles, 4; flush, 60%; and purge time, 60 sec. Finally, SPLE extracts (1.0 mL) were evaporated under nitrogen steam until dryness and re-dissolved in 1.0 mL of 5.0 mM of ammonium acetate in MeOH, which was the organic solvent used in the liquid chromatography mobile phase. Low recoveries (<20%) were obtained with all the solvents tested (Fig. 2), probably as a result of a high matrix effect. Nevertheless, MeOH was chosen as the solvent for the extraction process since it presented higher recoveries for all the pesticides tested and lower intra-day precisions. The influence of temperature (60ºC, 80ºC and 100ºC) in the extraction step was also studied in triplicate. As shown in Fig. 3, recoveries increased and standard deviation decreased (>25% for all compounds) from 60ºC to 80ºC. However, at 100ºC, a decrease of this parameter was observed, which can be explained by the possible degradation of compounds at high temperature. Therefore, the selected temperature was 80ºC.
Since the ion fragmentation of the mass spectrometer interface used Electrospray Ionisation (ESI), and due to the fact that ion suppression and ion enhancement originating from matrix effect is common in this ESI technique, the phenomenon was studied in the sediment, as recommended by Vanatta and Coleman (2007). Hence, the peak area obtained when a mixture of pesticide solution of 20 µg L⁻¹ (As) was injected in the UPLC-MS/MS was compared to the peak area of a spiked sediment sample (Ass) at the same level of concentration, as well as to the peak area of a non-spiked sediment sample (Anss), according to the following equation:

\[
\text{ME (\%)} = \frac{(\text{Ass}-\text{Anss})}{\text{As}} \times 100
\]

Percentages of matrix effect (ME) lower or higher than 100% were observed when ion suppression and enhancement occurred, respectively; and in the absence of matrix effects, ME should be equal to 100%. Results showed that, for the target pesticides, ME ranged from 10 to 17%, indicating a strong signal suppression possibly due to sediment constituents. Hence, a clean-up step should be added to the experimental procedure so as to improve the results and reduce matrix effect. Therefore, a new set of experiments was performed with the addition of the clean-up step. Three different clean-up techniques were tested in triplicate: (1) in-cell clean-up with Florisil, (2) on-line SPE with Oasis® HLB cartridges, and (3) a combination of both techniques, in-cell clean-up followed by on-line SPE. In (1), Florisil (0.5 g) was placed at the bottom of the ASE cell, which was filled with the homogenate of
sediment and dispersant agent. In (2), 1.0 mL of the SPLE extract was diluted up to 20 mL with ultrapure water, and then injected in an on-line SPE-UPLC-MS/MS system. In (3), the combination of these two techniques was tested. As shown in Fig. 4, low recoveries (<40% for all compounds) were observed in (1). Moreover, similar results were obtained in (2) and (3), except in the case of λ-cyhalothrin, which seemed to be retained in the Florisil (recovery <40%). The optimisation of these conditions came as a compromise because the selected pesticides exhibit very different physico-chemical properties. Accordingly, on-line SPE was selected as a suitable clean-up technique for marine sediment samples.

The adopted conditions of the SPLE-on-line SPE procedure for pesticide extraction and purification of sediment samples were: solvent extraction, MeOH; pressure, 1,500 psi; temperature, 80ºC; static time, 3 min.; number of cycles, 4; dispersant agent, diatomaceous earth (0.25 g); clean-up, on-line SPE.

![Graph showing recovery percentages of different compounds using different clean-up techniques](image)

**Fig. 4** Responses of the different sediment clean-up techniques. Results are expressed as the mean ± standard deviation (N=3).

After using the above-described ASE-on-line SPE procedure to improve the results and to reduce matrix effect, ME ranged between 73 to 95%, thus demonstrating the clean-up efficacy of the selected method. For this ME calculation, a spiked aqueous extract (20 µg L⁻¹) was used as standard mixture stock solution (As) and the quantitation of the compounds was carried out using an external matrix calibration.
Macrophytes

Sample preparation optimisation for macrophytes was also based on the SPLE technique. The same stepwise optimisation approach was followed. For the estimation of recoveries, a mixture of freeze-dried *Ulva* spp. and *Z. noltii* homogenised tissues (0.1 g) was spiked with a standard mixture stock solution at a concentration level of 50 ng g\(^{-1}\) dw for all the compounds, and carefully mixed by means of a glass rod in order to obtain a realistic contact between the sample and the added compounds. Then, for solvent optimisation, MeOH, acetonitrile, and a mixture of acetone:MeOH (50:50 v/v) were tested in triplicate, being MeOH the solvent selected due to the low intra-day precision generally obtained (Fig. 5). Different temperatures (40ºC, 60ºC, 80ºC and 100ºC) were tested as well. Higher recoveries were observed for almost all the pesticides at 40ºC (Fig. 6). Nevertheless, bentazon and penoxsulam presented better responses at 60ºC. In order to remove macrophyte pigments, different sorbents were tested for the in-cell clean-up step, such as neutral alumina, basic alumina, silica, PSA, and Florisil, as was an extraction cell without sorbent. The choice of the correct sorbent is critical since it controls the selectivity, affinity and capacity of an effective extraction. Hence, neutral alumina was the chosen sorbent as it allowed higher recoveries in most of the tested pesticides (Fig. 7). Two exceptions were observed, atrazine and terbuthylazine, which presented better responses with basic alumina.
The adopted conditions of the ASE in-cell clean-up procedure for macrophyte samples were: solvent extraction, MeOH; pressure, 1,500 psi; temperature, 40ºC; static time, 3 min.; number of cycles, 3; dispersant agent, diatomaceous earth (0.02 g); clean-up, in-cell with neutral alumina (0.65 g).

Aquatic animals

Analytical method optimisation for animals was based on the SPLE technique as well. An extra dispersant agent, silica (1.25 g), was introduced simultaneously with diatomaceous earth (0.125 g) in the preparation of the sample in order to reduce lipid interference in the analysis. For recovery determination, a mixture of freeze-dried *N. diversicolor* and *S. plana* homogenised tissues (0.5 g) were spiked (50 µL) with a standard mixture stock solution at a concentration level of 100 ng g⁻¹ dw for all the compounds, except for λ-cyhalothrin, which was at a concentration level of 2,000 ng g⁻¹ dw. The influence of the extraction solvent was tested as MeOH, acetonitrile, and a mixture of acetone:MeOH (50:50 v/v), being MeOH the solvent selected since it presented higher recoveries for all pesticides (Fig. 8). Different temperatures (40ºC, 60ºC, 80ºC and 100ºC) were also tested. Higher recoveries were observed at 40ºC for all pesticides except for penoxsulam, which presented a
better response at 60ºC (Fig. 9). To isolate the lipid fraction from the extracts and reduce interferences, different sorbents for the in-cell clean-up step were tested, namely neutral alumina, basic alumina, silica, PSA, and Florisil, as was an extraction cell without sorbent. As shown in Fig. 10, the best results were obtained with Florisil, as higher recoveries were observed in all pesticides. Additionally, the number of cycles (2 to 6 cycles) of the selected solvent (MeOH) in the extraction process was also tested. For almost all pesticides, higher recoveries were observed with 3 cycles (Fig. 11). However, atrazine and terbuthylazine presented better recoveries after 4 cycles of solvent.

The adopted conditions of the ASE in-cell clean-up procedure for animals tissues were: solvent extraction, MeOH; pressure, 1,500 psi; temperature, 40ºC; static time, 3 min.; number of cycles, 3; dispersant agent, 1.25 g of silica and 0.25 g of diatomaceous earth; clean-up, in-cell with Florisil (0.7 g).
On-line SPE-UPLC-MS/MS

The analyses of the target pesticides in all the studied matrices were performed by on-line SPE-UPLC-MS/MS. The clean-up procedure used in this system comprised two Oasis® HLB HP 20 µm columns (2.1 × 30 mm), which were directly connected, thus allowing a simultaneous clean-up (SPE column 1) and equilibration (SPE column 2) system in a shorter analysis time. This validated procedure, previously developed at IAREN (Water Institute of the Northern Region, Portugal) was performed as follows: conditioning SPE column 1 using ultrapure water and methanol (95:5 v/v) for 12 min., and then loading column 1 (1.0 mL min⁻¹) with the SPLE extract (diluted 1:20 with ultrapure water) using a 5.0 mL syringe and a 5.0 mL loop. In this technique, while analytes were retained on column 1, interferences were sent to waste and column 2 was equilibrated for 12 min. After this, the analytes were eluted from column 1 using the same mobile phase used in liquid chromatographic separation. Then, after the 12 min., the valve was switched back to the loading position and the next sample was loaded on column 1 (1.0 mL min⁻¹).

For chromatographic separation, samples were directly and automatically injected (injection loop: 5.0 mL) in a UPLC-MS/MS - “Inlet” (Waters Acquity UPLC) supplied with an Acquity UPLC® HSS T3 column of 1.8 mm, 2.1 × 150 mm, at 40 ± 1°C. The use of a shorter analytical column allowed reducing the chromatographic time while maintaining good efficiency in compound separation, thus increasing sample throughput. The mobile phase containing 5.0 mM of ammonium acetate in ultrapure water (A) and 5.0 mM of ammonium acetate in methanol (B) eluted at 0.3 mL min⁻¹ for 12 min. according to the following gradient conditions: started with 5% of (B), increased to 100% of (B) in 5 min. and continued in this percentage for 3 min. Then, it readjusted to the initial conditions in 2 min., and equilibrated in 2 further min.
The mass spectrometer (Waters TQD triple-quadrupole), equipped with an electro spray interface, operated in both negative (for bentazon, \(\lambda\)-cyhalothrin and penoxsulam) and positive (for atrazine, azoxystrobin and terbuthylazine) ion modes. Instrumental control and data acquisition and evaluation were carried out using MassLynx 4.0 software (Waters). The relevant instrumental conditions are shown in Table 7. Other important parameters were as follows: capillary voltage, 3,500V; source temperature, 140\(^\circ\)C; desolvation temperature, 350\(^\circ\)C; extractor voltage, 3V; RF lens, 0.2V. Nitrogen was used as the nebulizing and desolvation gas.

\[\text{Table 7} \quad \text{Retention times (RT) and MS/MS characteristics of the studied pesticides. The ion used for quantification is presented in bold.}\]

<table>
<thead>
<tr>
<th>ESI</th>
<th>RT (min)</th>
<th>Precursor ion ((m/z))</th>
<th>Product ions ((m/z))</th>
<th>Cone (V)</th>
<th>Collision energy (eV)</th>
<th>MRM ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrazine</td>
<td>PI</td>
<td>7.25±0.5</td>
<td>216 (M+H(^-))</td>
<td>174, 103</td>
<td>42</td>
<td>22, 32</td>
</tr>
<tr>
<td>Azoxystrobin</td>
<td>PI</td>
<td>5.86±0.5</td>
<td>404 (M(^+)H(^-))</td>
<td>372, 329</td>
<td>34</td>
<td>16, 34</td>
</tr>
<tr>
<td>Bentazon</td>
<td>NI</td>
<td>4.13±0.5</td>
<td>239 (M(^+))</td>
<td>133, 175</td>
<td>50</td>
<td>18, 24</td>
</tr>
<tr>
<td>-Cyhalothrin</td>
<td>NI</td>
<td>6.76±0.5</td>
<td>448 (M(^+))</td>
<td>420, 385</td>
<td>16</td>
<td>6, 10</td>
</tr>
<tr>
<td>Penoxsulam</td>
<td>NI</td>
<td>4.90±0.5</td>
<td>481 (M(^+))</td>
<td>179, 81</td>
<td>60</td>
<td>24, 32</td>
</tr>
<tr>
<td>Terbuthylazine</td>
<td>PI</td>
<td>6.19±0.5</td>
<td>230 (M(^+)H(^-))</td>
<td>174, 78</td>
<td>38</td>
<td>18, 28</td>
</tr>
</tbody>
</table>

Quality assurance

Multiple-reaction monitoring (MRM) was chosen as data acquisition mode because of its high sensitivity and selectivity. According to an European decision concerning the performance of analytical methods, four identification points (one precursor ion and two product ions) were recorded for the identification and confirmation of each target pesticide by UPLC-MS/MS analyses (European Commission, 2002). Retention times and MRM ratios were used as criteria for the identification of compounds to avoid false positive results and overestimation. In compliance with the abovementioned decision, variations between the retention time observed both in the sample and in the standard should be lower than 2%; regarding the MRM ratio, the relative abundance of MRM transitions in the sample should not differ by more than 20% (ratio 1-2), 25% (ratio 2-5), 30% (ratio 5-10) or 50% (ratio 10-100).
from that MRM ratio observed in the injected standard. The MRM chromatograms of spiked sediment (A), macrophyte (B) and organism (C) samples obtained after SPE extraction and clean-up, followed by on-line SPE-UPLC-MS/MS separation and quantification are shown in Figs 12 to 14.

Fig. 12 Chromatogram of spiked sediment (50 ng g⁻¹ of each compound).

A: azoxystrobin, B: atrazine, C: terbuthylazine, D: bentazon, E: λ-cyhalothrin,
F: penoxsulam.

Fig. 13 Chromatogram of spiked macrophytes (50 ng g⁻¹ of each compound).

A: azoxystrobin, B: atrazine, C: terbuthylazine, D: bentazon, E: λ-cyhalothrin,
F: penoxsulam.
Method validation

The adopted analytical methodology was validated using spiked sediment, macrophyte and organism material. Linear range (LR), linearity ($r^2$), method detection limit (MDL), method quantitation limit (MQL), extraction recovery (R) and intra-day precision (expressed as the relative standard deviation (RSD)) were determined. LR was evaluated using a 10-point calibration curve. Linearity was verified according to the criteria established by ISO 8466-1 (1990). MDL and MQL were calculated as $3 \times \frac{S_y}{x/b}$ and $10 \times \frac{S_y}{x/b}$, respectively, where $S_y/x$ is the standard error of the estimated curve and $b$ the slope of the calibration curve. Accuracy and precision were evaluated at two different concentrations, $3 \times$ MQL and $10 \times$ MQL, according to European Commission (2002). Recoveries (%) were assessed by comparing the matrix calibration chromatograms with the spiked samples, and RSDs were calculated using six replicates analysed on the same day and by the same analyst.

Uncertainty of the analytical method was estimated on the basis of in-house validation data according to EURACHEM/CITAC Guide for all compounds at two

![Fig. 14 Chromatogram of spiked organisms (100 ng g⁻¹ of each compound, except for λ-chyalothrin, which was at a concentration level of 2,000 ng g⁻¹). A: azoxystrobin, B: atrazine, C: terbuthylazine, D: bentazon, E: λ-cyhalothrin, F: penoxsulam.](image-url)
spiked levels (EURACHEM, 2012). The main sources of uncertainty were identified and quantified, and combined uncertainty \( (u_c) \) was calculated as follows:

\[
u_c(y) = \sqrt{u_1^2 + u_2^2 + u_3^2}
\]

where \( u_1 \) is the uncertainty associated with the interpolation of the sample reading in the calibration curve that reflects the influence of the lack of fit of the regression plot on the analytical result, \( u_2 \) is the uncertainty indicated by the supplier of the commercial standards used, and \( u_3 \) is the uncertainty associated with variability/precision of the method. The expanded uncertainty \( (u_{exp}) \) was estimated using the coverage factor \( k = 2 \) for a level of confidence of 95%, as follows:

\[
u_{exp} = k \times u_c
\]

Results and discussion

Method validation

Analytical figures of merit by target pesticide are shown in Table 8: determination coefficients \((r^2)\) were higher than 0.9912 for all compounds at the adequate range. MQL ranged between 1.2 (atrazine) and 16.5 (\(\lambda\)-cyhalothrin) ng g\(^{-1}\) dw for sediment, 6.8 (terbuthylazine) and 17 (penoxsulam) ng g\(^{-1}\) dw for macrophytes, and 11 (azoxystrobin) and 290 (\(\lambda\)-cyhalothrin) ng g\(^{-1}\) dw for animals. Results demonstrated that the methods achieved satisfactory recoveries (>60%) for all the pesticides, except for bentazon and penoxsulam, which presented recoveries between 49 and 57% for macrophytes (Table 8). An overview of all recovery values indicated a range of 50-125% for the lowest-level and of 49-135% for the highest-level concentrations. In general, the developed methods presented intra-day precisions ≤15%, which are within the range of the acceptance criteria of the European Quality Control Guidelines (RSD <20%, e.g., European Commission, 2013). However, \(\lambda\)-
cyhalothrin presented an intra-day precision of 22% for organism samples. Yet, pyrethroids pesticides, as non-ionised compounds, are known to be par excellence detected by gas chromatography (Wang et al., 2009; Shi et al., 2012).

The relative expanded uncertainties of the target pesticides in all the studied matrices at the two considered concentrations were lower than 30% (30% for λ-cyhalothrin in organism samples) for all compounds.

Comparison with previous analytical methodologies

The proposed novel methodologies based on SPLE on-line SPE-UPLC-MS/MS for the determination of residues of atrazine, azoxystrobin, bentazon, λ-cyhalothrin, penoxsulam and terbutylazine in sediment, macrophyte and organism matrices showed important improvements in comparison with the previously reported methods. For instance, SPLE allowed the simultaneous extraction and clean-up of
the samples, and automatic on-line SPE coupled to the liquid chromatographic system reduced sample handling, thus avoiding error. These methods minimised solvent use, analysis time and waste generation, according to Green Chemistry principles. Regarding UPLC-MS/MS determination, the fact that a short analytical column was used increased speed with superior resolution and sensitivity, including the case of λ-cyhalothrin. Moreover, low method quantitation limits (trace levels) using a small amount of sample (in the order of milligrams) were achieved. The quantification limits are in line with recent studies found in literature: considering sediments, atrazine was measured in concentrations of 2.0 and 1.5 ng g\(^{-1}\) dw by Allinson et al. (2015) in Australian urban and peri-urban wetlands and by Magnusson et al. (2013) in Australian rivers, respectively; azoxystrobin was also measured by Allinson and collaborators in a concentration of 7.0 ng g\(^{-1}\) dw, and λ-cyhalothrin was measured in concentrations of 11.1 and 11.7 ng g\(^{-1}\) dw by Weston et al. (2013) in the California's Central Valley and by Li et al. (2014) in Guangzhou (China), respectively. No field studies were found that measured the current levels of the pesticides selected for the present study in macroalgae, aquatic plants, or clams. Nevertheless, it was possible to conclude that the proposed methodology allows the successful determination of the six selected pesticides at trace levels in three different marine matrices.

Pesticides in the Mondego estuary

No pesticide concentrations above their respective method quantification limits were measured in sediments and in aquatic plants collected in the Mondego estuary in August 2014. However, terbuthylazine was found in the macroalgae Ulva spp. (108 ng g\(^{-1}\) dw), and all the pesticides prospected were found above their respective method quantification limits in the 3+ size class of the bivalve S. plana: atrazine, 48
ng g\(^{-1}\) dw; azoxystrobin, 64 ng g\(^{-1}\) dw; bentazon, 33 ng g\(^{-1}\) dw; \(\lambda\)-cyhalothrin, 2,531 ng g\(^{-1}\) dw; penoxsulam, 50 ng g\(^{-1}\) dw; and terbuthylazine, 44 ng g\(^{-1}\) dw.

**Conclusions**

Analytical methodologies for the simultaneous analysis of atrazine, azoxystrobin, bentazon, \(\lambda\)-cyhalothrin, penoxsulam and terbuthylazine in sediments, macrophytes and animals were successfully developed and validated. Adequate accuracy and satisfactory precision were achieved by the proposed methods. Moreover, sensitivity and selectivity allowed low method quantitation limits using small amounts of sample (in the order of milligrams). Other advantages are automaticity (single-step pesticide extraction and clean-up, as well as clean-up and determination), fastness (total analysis time of 20 min.), and low consumption of solvents and low waste generation, in compliance with Green Chemistry principles.

All the six prospected pesticides were measured above their respective method quantification limits in the Mondego estuary: atrazine (in bivalves), azoxystrobin (in bivalves), bentazon (in bivalves), \(\lambda\)-cyhalothrin (in bivalves), penoxsulam (in bivalves) and terbuthylazine (in macroalgae and bivalves). Therefore, this innovative analytical methodology could also be applied to food safety analyses.
SEASONAL AND SPATIAL OCCURRENCE AND FATE OF PESTICIDES IN THE MONDEGO ESTUARY (PORTUGAL)

The development of new and significant analytical methodologies to measured multi-residue pesticides in complex matrices allow us to studied the seasonal and spatial occurrence and fate of atrazine, azoxystrobin, bentazon, λ-cyhalothrin, penoxsulam and terbuthylazine in the Mondego estuary.

Abstract

The seasonal (March and August) and spatial occurrence and fate of five current-use pesticides (azoxystrobin, bentazon, λ-cyhalothrin, penoxsulam and terbuthylazine) plus legacy atrazine were investigated in the Mondego estuary (Portugal). The novelty of this study is the use of complex marine matrices to prospect these pesticides. Hence, the above-mentioned analytical methodology was used to measure the pesticides in sediment, macroalgae, aquatic plants, aquatic worms and bivalves. Quantified concentrations were determined mostly during summer in agreement with the Lower Mondego (agricultural area located upstream the estuary) pesticide application period. Azoxystrobin presented the highest detection frequency and atrazine presented the second highest frequency, thus highlighting the need to include legacy pesticides in monitoring programmes in order to provide realistic pollutant assessment. Bentazon concentrations in surface water were considerably higher than those reported for other countries, and also exceeded the European standard of 0.1 μg L⁻¹ for drinking water in all samples collected both in March and August. Animal species typically accumulated more pesticide residues than plant species. Thus, the present study highlighted the
widespread interest of bivalves as biomonitor organisms of pesticides under environmental conditions. It was possible to conclude that the age of *S. plana* determined clam susceptibility towards pesticide accumulation, with older animals being in general more vulnerable than younger ones. Azoxystrobin was the dominant pesticide bioaccumulated in *S. plana*, and it was measured in similar concentrations in both digestive gland and remaining tissue, suggesting that the use of digestive gland or whole body is equally valid to address the concentration of azoxystrobin in this species. Conversely, the other pesticides indicated higher digestive gland metabolic rates and rapid accumulation in other tissues. All the prospected pesticides were bioaccumulated by *S. plana*, leading us to consider that pesticides may not only cause adverse effects on the aquatic organism itself, but also pose a potential human health risk, since this is an edible species and is considered of economic interest. Concern is also expressed about edible seaweeds, since s-triazine pesticides were also measured in both *Ulva* spp. and *G. gracilis*. Acknowledging these concerns, developing and establishing allowable pesticide tolerance values for edible seaweeds, fish, and crustaceans and molluscs (shellfish) is recommended.

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**Materials and Methods**

**Study area**

The Mondego estuary (6.4 Km²) is located in southern Europe, in the central coast of Portugal and was classified as a mesotidal well-mixed estuary with irregular river discharges by Bettencourt et al. (2004). The estuary drains a hydrological basin of 6,659 km² (APA, 2015) and its most downstream part is divided into two channels ([Fig. 15](#)). The northern channel is deeper (4-8 m deep at high tide), with regularised
banks for navigation and harbour facilities, while the southern channel is shallower (2-4 m deep at high tide) and characterised by large areas of intertidal flats exposed during low tide. The southern channel receives, besides the water coming from River Mondego, water from River Pranto, whose discharge is artificially controlled by a sluice located about 3 km upstream its mouth, as well as water coming from an important creek (Esteiro dos Armazéns). In the valley of River Pranto, around 80% of the crops come from rice fields. Close to the estuary, both in River Pranto and Esteiro dos Armazéns, several fish farms develop their activities. Also, a processing industry of vegetable and fish sub-products is located in the bank of the creek. Sampling was carried out at spatially representative stations along the south channel, as well as in River Pranto, covering a total of four sampling points (Fig. 15). Station A corresponds to a high depositional zone which is not in the leading line of the water (40°07,815′N; 8°49,692′W), station B is situated in the mouth of Esteiro dos Armazéns (40°07,308′N; 8°50,515′W), Station C is located in River Pranto (40°07,048′N; 8°49,692′W) and station D is the most upper stream station (40°07,342′N; 8°49,036′W). The sampled area comprises the richest habitats of the Mondego estuary, ensuring both a high primary and secondary macrobenthic production and the biodiversity of this natural system (e.g., Dolbeth et al. 2011; Rodrigues and Pardal, 2015).

Fig. 15 Map of the Mondego estuary showing the study area. A, B, C and D mark the location of the sampling stations.
Sample collection

Samples were collected during low tide at the mentioned study area during the months of March and August 2014, representing the lowest and highest probability of presence of pesticide residues in the Mondego estuary, according to the 2014 pesticide application schedule for the Lower Mondego. During collection, samples were stored in new amber glass or aluminium extrusion containers and transported to the laboratory in 12V car refrigerators. In each sampling station, surface water samples (≈1.0 L) were collected in precleaned amber glass bottles in the middle of the water-course. Intertidal sediment samples were randomly collected (top 5 cm) in quadruplicate within an area of 100 m² in each sampling station using a stainless steel core (5 cm Ø). Water (unfiltered) and sediment samples (three replicates of each sampling point) were frozen immediately upon arrival to the laboratory. In order to consider the potential effects of organic material on the bioavailability of pesticides to aquatic organism, the organic matter content of the sediments was determined. Thus, the fourth replica of sediment was oven-dried to constant weight at 60ºC (Raypa) to obtain dry weight, and then burned at 450ºC (Nüve MF110) for eight hours to obtain organic matter content. Before being frozen, all the collected macrophytes (*Ulva* spp., *G. gracilis, F. vesiculosus, Z. noltii, S. maritima* and *S. maritimus*) were rinsed briefly in running tap water and gently scrubbed with paper towels to remove most surface microbial and epiphytic organisms, and then aquatic plants (except *Z. noltii*) were separated in root-rhizome, stems, and leaves. The collected aquatic worms and bivalves were maintained for gut clearing purposes as described in the previous study. After 72 hours, the worms were dried with paper towels and frozen. Concerning bivalves, *S. plana* were maintained in these conditions for 96 hours for depuration purposes. After depuration, *S. plana* were measured using an electronic digital caliper and separated in five size classes (0-1, 1-2, 2-3, 3-4 and >4 cm). The most abundant size class in the study area was 3+,
and therefore, this size class was dissected and dried with paper towels, and weighed (total wet weight (ww)). The dissected animals were then separated in digestive gland and remaining tissues, and the digestive gland was weighed. The weighed values were computed to quantify the degree to which total and digestive gland weights were related, and to determine what percentage of the total weight corresponds to the digestive gland. Since age may affect the bioaccumulation of chemicals in *S. plana* (e.g., Coelho et al., 2006), in the sampling campaign of August, size classes 1+, 2+, and 4+ were also collected, depurated, dissected, and frozen. Nevertheless, size classes 1+ and 2+ did not present enough material for further chemical analyses. Therefore, for these size classes, the bivalves collected in the sampling stations A and B (downstream) were mixed, as well as those collected in the sampling stations C and D (upstream). Moreover, organisms of the size class 4+ were only found in stations B and C. The frozen samples of macrophytes and animals were freeze-dried (UniEquip Unicryo MC-4L) and then grinded. After homogeneition, they were saved in new amber glass vials.

Analytical methods

For water samples, a flow injection SPE system was adopted for automatic determination of pesticide concentrations according to a method routinely used at IAREN. This method was based on on-line chelate complex formation of target pesticides and retention onto the surface of reversed-phase poly(divinylbenzene-N-vinylpyrrolidone) co-polymeric beads (Oasis® HLB 20 mm, 2.1×30 mm) and elution (1.0 mL min⁻¹) with a mixture of 5% methanol (MeOH, Chromasolv® HPLC-grade) and 95% ultrapure water (Millipore Direct MilliQ water system). The extraction procedure used 5.0 mL aliquots of the samples, which were previously filtered through a 0.45 μm membrane filtre.
Pesticide extraction and purification steps of sediment, macrophyte, and organism samples were based on the SPLE technique according to methods developed in the previous study. After extraction, clean-up, separation and quantification of the selected pesticides were performed by on-line SPE-UPLC-MS/MS as abovementioned.

Quality control

For water samples, to control the analytical reliability and assure the recovery efficiency and the accuracy of the analytical results, LR, \( r^2 \), MDL, MQL, R, and RSD were determined by target pesticide. Accuracy and precision were evaluated according to the European Commission (2002) as \( 10 \times MQL \), and RSD was calculated using 10 replicates analysed in the same day and by the same analyst (Table 9).

<table>
<thead>
<tr>
<th>Water Matrix</th>
<th>Atrazine LR (µg L(^{-1}))</th>
<th>Azoxystrobin LR (µg L(^{-1}))</th>
<th>Bentazon LR (µg L(^{-1}))</th>
<th>Cyhalothrin LR (µg L(^{-1}))</th>
<th>Peroxsulam LR (µg L(^{-1}))</th>
<th>Terbutylazine LR (µg L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>LR (µg L(^{-1}))</td>
<td>0.025-0.15</td>
<td>0.025-0.15</td>
<td>0.025-0.15</td>
<td>0.025-0.15</td>
<td>0.025-0.15</td>
<td>0.025-0.15</td>
</tr>
<tr>
<td>( r^2 )</td>
<td>0.9994</td>
<td>0.99969</td>
<td>0.9991</td>
<td>0.9993</td>
<td>0.9993</td>
<td></td>
</tr>
<tr>
<td>MDL (µg L(^{-1}))</td>
<td>0.005</td>
<td>0.0011</td>
<td>0.011</td>
<td>0.025</td>
<td>0.005</td>
<td>0.005</td>
</tr>
<tr>
<td>MQL (µg L(^{-1}))</td>
<td>0.015</td>
<td>0.0034</td>
<td>0.033</td>
<td>0.076</td>
<td>0.015</td>
<td>0.016</td>
</tr>
<tr>
<td>10 ( \times ) MQL (N=10) R (%)</td>
<td>79</td>
<td>67</td>
<td>70</td>
<td>88</td>
<td>71</td>
<td>85</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>6.1</td>
<td>5.6</td>
<td>7.9</td>
<td>23</td>
<td>8.6</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Statistical analysis

The Mann-Whitney U Test was the nonparametric test used to verify the statistical significance of the difference between pesticide concentrations in the digestive gland and the remaining tissue in 3+ size class S. plana, as well as between pesticide concentrations collected in March and August (whole organism). The Pearson correlation was the test used to quantify the degree to which total and digestive gland weights are related (N = 200). Statistical analyses were performed.
using the STATISTICA 7.0 software. A value equal or inferior to 0.05 was considered as level of statistical significance.

**Results**

Among the six prospected pesticides, only the fungicide azoxystrobin and the herbicide bentazon were detected in estuarine water. Concentrations ranged from the method quantification limits to 0.07 μg L\(^{-1}\) for azoxystrobin and to 3.4 μg L\(^{-1}\) for bentazon. Both pesticides presented seasonal differences, being azoxystrobin only found in August (in all sampling stations), and bentazon in both sampling periods (March and August) and in all sampling stations, with higher values mostly in August (River Pranto showed similar values: 2.8 and 2.8 μg L\(^{-1}\) in March and August, respectively). As shown in Fig. 16 A (March) and B (August), a spatial gradient was not evident. However, in general, higher values were measured at the River Pranto sampling station (station C).
Fig. 16 Pesticide levels found in water (●), sediment (●) and macroalgae (● for Ulva spp. and ● for Gracilaria gracilis) samples, as well as organic matter content of the sediment (●), by sampling station in the Mondego estuary, in March (A) and August (B) 2014.

The organic matter content in the intertidal sediment is shown in Fig. 16 A and B. Results indicate seasonal differences, with higher values presented in August. A spatial gradient is not fully evident, since similar values, between 3.8 and 4.8%, were determined in March in the study area. However, in August, a clear gradient was found, with increasing values from downstream (station A) to upstream (station D). Among the six prospected pesticides, only the herbicide atrazine and the fungicide azoxystrobin were measured in sediments. Concentrations ranged from their method quantification limits to 2.0 ± 0.21 ng g⁻¹ dw for atrazine and 3.9 ± 0.12
ng g\(^{-1}\) dw for azoxystrobin. Both pesticides presented seasonal differences, since they were only found in March and in sampling station A.

Atrazine and terbutylazine were the two pesticides measured above their respective method quantification limits in macroalgae. Atrazine presented a concentration of 20 ng g\(^{-1}\) dw in *G. gracilis* and terbutylazine in concentrations which ranged from their method quantification limit to 149 ng g\(^{-1}\) dw. Both pesticides presented seasonal differences, being only found in August. As shown in Fig. 16 B, terbutylazine was measured in stations B and C, with higher value at the River Pranto sampling station (station C).

No pesticide concentrations above their respective method quantification limits were measured in aquatic plants (in both sampling periods) or aquatic worms (in March). Due to laboratorial constraints, the aquatic worm samples collected in August were not analysed. However, all the pesticides prospected measured above their respective method quantification limits in the bivalve *S. plana*. Concerning the 3+ size class, the most abundant size of the study area, results are presented in Table 10. Within this size class, the digestive gland of *S. plana* represent 23% of the total weight of the organism, being both variables related by the following positive correlation ($r^2 = 0.618$, $N = 200$):

$$dgw (ww) = 0.19 \times tw (ww) + 0.040$$

where $dgw$ is the weight of the digestive gland and $tw$ is the total organism weight. For this size class (3+), the pesticide concentration of the whole organism was attained by multiplying the result of the digestive gland by 0.23, and the result of the remaining tissue by 0.77. If one of the results, from the digestive gland or the remaining tissue, was below their respective method quantification limit, the value of that limit was used for the calculation (Table 10).
The dominant pesticide in 3+ size class bivalves was azoxystrobin, with quantification frequencies of 100% in both sampling periods. No statistical significance of the differences between azoxystrobin concentrations of the digestive gland and of the remaining tissue was found \((P = 0.534)\), or between azoxystrobin concentrations from March and August \((P = 0.343)\). Nevertheless, for the other prospected pesticides, seasonal influence was observed since they were only determined above their respective method quantification limits in the samples collected in August. Notably, in this sampling period, *S. plana* collected in station B (mouth of the Esteiro dos Armazéns) presented contamination by all the pesticides prospected, and in stations C and D, besides azoxystrobin, atrazine was also measured. Concerning 1+ and 2+ size classes, which were collected only in August, azoxystrobin was the only pesticide measured above its method quantification limit: 1+ (stations A/B: 20 ng g\(^{-1}\) dw, stations C/D: 17 ng g\(^{-1}\) dw) and 2+ (stations A/B: 20 ng g\(^{-1}\) dw, stations C/D: 23 ng g\(^{-1}\) dw). Regarding the 4+ size class, which was collected only in stations B and C (August), four pesticides measured above their respective method quantification limits: atrazine (station B: 83 ng g\(^{-1}\) dw, station C: 23 ng g\(^{-1}\) dw), azoxystrobin (station B: 78 ng g\(^{-1}\) dw, station C: 21 ng g\(^{-1}\) dw), penoxsulam (station B: 124 ng g\(^{-1}\) dw), and terbutylazine (station B: 88 ng g\(^{-1}\) dw).  

To conclude, among all the prospected pesticides, azoxystrobin was the most abundant residue in all size classes of *S. plana*, followed by atrazine. Additionally,
bivalves collected in station B (August) presented bioaccumulation of all the pesticides prospected, as well as the highest concentration of pesticides of the study area, with a maximum of 2,531 ng g\(^{-1}\) dw for λ-cyhalothrin (whole organism data).

**Discussion**

Research carried out under this study established both seasonal and spatial occurrence and fate of current-use (azoxystrobin, bentazon, λ-cyhalothrin, penoxsulam, and terbuthylazine) and banned legacy (atrazine) pesticides on a temperate estuarine system (Mondego estuary). All the prospected pesticides were detected above their respective method quantification limits in the Mondego estuary: atrazine (in sediments, macroalgae and bivalves), azoxystrobin (in water, sediments and bivalves), bentazon (in water and bivalves), λ-cyhalothrin (in bivalves), penoxsulam (in bivalves), and terbuthylazine (in macroalgae and bivalves). Regarding the herbicide atrazine, which was officially used in the region up to 2007, the gathered results demonstrated its potential to persist in the environment since it was still found in 2014. This pesticide was expected to adsorb to sediments (Koc 64-546), and thus measured (2.0 ng g\(^{-1}\) dw) above its method quantification limit in sediments of sampling station A (low organic matter content), but only in March (see Fig. 16 A). The Seybold et al. (1999) study corroborates our result, since these authors demonstrated atrazine’s great potential for sediment accumulation under cold temperatures and in low adsorption capacity sediments, i.e., low organic matter content. On the other hand, atrazine was found in macroalgae (*G. gracilis*) in August in a single sampling station, the one with the highest organic matter content of the study area (station D, see Fig. 16 B). Therefore, results suggested a seasonal and spatial (depending on organic matter content) influence on atrazine mobility. Atrazine was commonly measured in sediment samples, being similar maximum
concentrations of 2.0 and 1.5 ng g\(^{-1}\) dw reported by Allinson et al. (2015) in Australian urban and peri-urban wetlands and by Magnusson et al. (2013) in Australian rivers in north Queensland, respectively. However, much higher values, 392 and 940 ng g\(^{-1}\) dw, were found in sediments of River Sava, which is a Serbian Danube tributary (Radović et al., 2015) and in sediments of the Nigerian River Owan (Ogbeide et al., 2015), respectively. The Log Kow of 2.6 characterizes the low liposolubility of atrazine. However, its BCF between \(<0.27\) and 100, indicates that atrazine would potentially generate low to moderate bioaccumulation and biomagnification processes. Hence, atrazine was measured in \(S.\ plana\) tissues in the samples collected in August, presenting a maximum concentration of 83 ng g\(^{-1}\) dw (4+ size class). A spatial influence was also observed in the present study, since atrazine was only measured in animals collected in stations B, C and D, which presented the highest organic matter content of the study area. The bivalve \(S.\ plana\) is a predominantly surface deposit feeder of sedimentary organic matter and, thus, organism concentrations of atrazine in the study area appear to be linked to the organic matter content of the sediment. Organism concentrations of atrazine appear to also be linked to time of life, since only higher size classes (3+ and 4+ years old) presented atrazine bioaccumulation. Atrazine increased in \(S.\ plana\) from 48 (3+) to 83 ng g\(^{-1}\) dw (4+) in station B, and from 19 (3+) to 23 ng g\(^{-1}\) dw (4+) in station C, indicating great accumulation by older organisms. The potential of atrazine bioaccumulation in bivalve tissues was also demonstrated by Jacomini et al (2006).

The fungicide azoxystrobin was found dissolved in the water only in August, but along all the study area. This presence seems to be linked to the high application period of azoxystrobin in the Lower Mondego and to its very high potential to move in runoff (Deb et al., 2010; Long et al., 2005). In 2014, around 4,100 ha of rice crops were subjected to azoxystrobin treatment at the end of July/beginning of August (technical information of the Regional Direction of Agriculture and Fisheries of the
Centre of Portugal). Maximum concentration (0.07 µg L\(^{-1}\)) was measured in the River Pranto station (station C), which is in line with the high percentage of rice crop production of the Pranto valley (80%). When azoxystrobin reaches the south channel, it possibly undergoes a dilution effect and, thus, low concentrations were measured downstream (stations A and B, both with 0.05 µg L\(^{-1}\)). Considering the upper station (station D), the value of 0.04 µg L\(^{-1}\) suggests a prior dilution due to the high flow of River Mondego. Azoxystrobin maximum concentrations reported for US streams and ponds by Reilly et al. (2012) and for US lakes and creeks in high elevation national parks by Keteles (2011) were in the same order of magnitude (both with 0.06 µg L\(^{-1}\)) of that reported in the present study (0.07 µg L\(^{-1}\)). However, most worldwide studies reported higher maximum concentrations: 0.16 µg L\(^{-1}\) in amphibian habitat ponds of seven US States by Smalling et al. (2012) and in the Ria Formosa Lagoon (Portugal) by Cruzeiro et al. (2015), 0.18 µg L\(^{-1}\) in Australian urban and peri-urban wetlands by Allinson et al. (2015), 0.19 µg L\(^{-1}\) in Brazilian (Neópolis) surface water by Filho et al. (2010), 0.51 µg L\(^{-1}\) in Danish (Island of Funen) streams by Rasmussen et al. (2012), 0.54 µg L\(^{-1}\) in French (Lyon) streams by Rabiet et al. (2010), 1.1 µg L\(^{-1}\) in steams of 13 US States by Battaglin et al. (2011), 1.4 µg L\(^{-1}\) in Danish surface water by Jørgensen et al. (2012), 2.4 µg L\(^{-1}\) in Vietnamese (lower Mekong river delta) surface water by Chau et al. (2015), 11 µg L\(^{-1}\) in German (Lower Saxony) streams by Liess and von der Ohe (2005), and 29.7 µg L\(^{-1}\) in German (Lower Saxony) streams by Berenzen et al. (2005). Azoxystrobin was also measured (3.9 ng g\(^{-1}\) dw) above its method quantification limit in intertidal sediments (only in March), in a single sampling station (station A), suggesting a seasonal and spatial influence in azoxystrobin mobility. Photodegradation as an important elimination process of azoxystrobin is well studied (this project, Rodrigues et al., 2013c), and could explain its presence in the sediments of the estuary only in March. Nonetheless, its unexpected presence in March (application was at the end of July/beginning of August) was possibly due to the agricultural practice of filling the
rice fields with water during around the three months prior to the beginning of the production season, since, in 2014, Pranto valley rice fields were discharged from this wash practice during the month of March (technical information). Regarding the spatial occurrence of azoxystrobin in sediments, fine particulate matter and particle-bound residues are preferentially transported within estuaries to areas of high deposition (as station A), and according to Singh and Singh (2010), azoxystrobin is more persistent in non-flooded soils than in flooded soils. Azoxystrobin was also reported in sediment samples by Allinson et al. (2015) and Smalling et al. (2012) who measured higher values in Australian urban and peri-urban wetlands (average concentration 7.0 ng g\(^{-1}\) dw) and in amphibian habitats and ponds of seven US States (maximum concentration 12.6 ng g\(^{-1}\)), respectively. Azoxystrobin is not expected to bioaccumulate due to its low octanol-water coefficient and BCF (Log K\(_{ow}\) 2.5, BCF 21). However, this residue was measured in a 100% frequency in collected *S. plana* bivalves, suggesting that predictions of the K\(_{ow}\) and BCF, which can be predicted from K\(_{ow}\) via computer programmes, have noteworthy limitations.

Concerning the 3+ size class, which was the only size class analysed in both sampling periods, concentrations were statistically similar in March and August, indicating no seasonal variation. Azoxystrobin concentrations in the digestive gland were also statistically similar to the remaining tissue concentrations. In March, a spatial gradient was not evident (mean value of 27 ng g\(^{-1}\) dw). However, in August, the mean value of azoxystrobin concentrations in stations A, C and D (17 ng g\(^{-1}\) dw) was much lower than the concentration measured in station B (64 ng g\(^{-1}\) dw). The latter value was possibly due to a point source contamination linked to a reduced time period of azoxystrobin bioaccumulation by *S. plana*. For instance, Lazartigues et al. (2013) studied multi-residue bioaccumulation and decontamination kinetics in two freshwater fish species (*P. fluviatilis* and *C. carpio*) and determined that azoxystrobin reached a steady state within 6 days in muscle tissues of both species. Regarding 1+ and 2+ size classes, chemical analyses were performed in mixed
tissue samples collected in stations A and B (downstream), as well as in stations C and D (upstream). Hence, to better understand the influence of age in the bioaccumulation process, a concentration mean value of stations A and B, and C and D were determined for the 3+ size class (downstream: 40 ng g\(^{-1}\) dw, upstream: 18 ng g\(^{-1}\) dw). Thus, downstream azoxystrobin bioaccumulation was 20 (1+), 20 (2+), 40 (3+), 78 (4+) ng g\(^{-1}\) dw, whereas upstream bioaccumulation was 17 (1+), 23 (2+), 18 (3+), 21 (4+) ng g\(^{-1}\) dw. Results suggest that, at highly contaminated sites, the capability of *S. plana* to bioaccumulate azoxystrobin is higher in older organisms. However, this is not so evident in less contaminated sites.

Regarding bentazon, which is a non-volatile herbicide (Henry's law constant of \(2.2 \times 10^{-9}\) atm-cu m mol\(^{-1}\), [TOXNET database (2015)](https://toxnet.nlm.nih.gov/tceis/home.html)) with low persistence in sediments (Koc 13-176) and highly soluble (500 mg L\(^{-1}\)) in water, it was found dissolved in the water phase in both sampling periods, March (mean value of 1.2 μg L\(^{-1}\)) and August (mean value of 2.7 μg L\(^{-1}\)), with a maximum concentration of 3.4 μg L\(^{-1}\) (measured in August in station B). Bentazon exceeded the European standard of 0.1 μg L\(^{-1}\) for drinking water in all samples in both sampling periods. An overview of pesticide concentration (2010 to 2012) in 14 streams in 4 catchments located on Sjaelland, Denmark, was reported by [McKnight et al. (2015)](https://doi.org/10.1002/2014JD021941), and a maximum bentazon concentration of 0.016 μg L\(^{-1}\) was measured. Bentazon was also reported in a US pesticide monitoring survey (in 2009) in North Dakota rivers, where the maximum concentration was 0.07 μg L\(^{-1}\) ([Johnson and Gray, 2009](https://doi.org/10.1002/2009JD012046)). All the concentrations measured in the present study were one or more orders of magnitude higher than those reported as maximum concentrations in the above mentioned studies. Bentazon was commonly used in the Lower Mondego for maize and rice weed control, which may involve direct application to the water in the latter case. Bentazon appears to be stable to hydrolysis and its presence in March was possibly due to the agricultural practice of filling the rice fields with water before the
beginning of the production season. It should be stressed that, in March, the highest value was measured in the River Pranto sampling station (station C). In theory, the small octanol-water partition coefficient (Log Kow 3.8) of bentazon precludes bioaccumulation processes. However, a BCF of 79 indicates that that would potentially generate low to moderate bioaccumulation potential, and thus it was determined (single measurement of 37 ng g\(^{-1}\) dw) in 3+ \textit{S. plana} tissues, but only in the remaining tissues (after excluding the digestive gland), which suggests that bentazon is rapidly metabolised by the digestive gland, the main metabolic site, and it is then accumulated in other tissues.

Possibly due to its very low solubility in water (0.005 mg L\(^{-1}\)) and high potential to be bioaccumulated (Log Kow 6.9), the pyrethroid insecticide \(\lambda\)-cyhalothrin was only found in the study area in \textit{S. plana} tissues in a very high concentration (single measurement of 3,200 ng g\(^{-1}\) dw in 3+ size class), but only in the remaining tissues (excluding the digestive gland). According to the peer review of the pesticide risk assessment of \(\lambda\)-cyhalothrin, MRLs of this pesticide, despite provisional, varies between 10 (e.g., birds’ eggs, poultry and ruminants muscle) and 200 (e.g., ruminants fat) ng g\(^{-1}\) (EFSA, 2014). Even though its carbon adsorption coefficient (Log Koc 5.5) is also high, which indicates preferential affinity to organic matter, no \(\lambda\)-cyhalothrin measurements were found in any sediment sample. This was possibly due to its short persistence both in water and sediments, as highlighted by Gu et al. (2007). On the other hand, bivalves’ ability to bioaccumulate lipophilic residues such as this insecticide is well known.

Penoxsulam is an herbicide used to control a wide range of weeds in rice crops. This pesticide is very soluble in water and its volatilisation from water surfaces is not expected (Henry’s Law constant of 1.1\(\times\)10\(^{-18}\) atm-cu m mol\(^{-1}\), TOXNET database (2015)), nor is adsorption to sediments (Koc 30). Despite its low potential to be bioaccumulated (Log Kow -0.354, BCF 3), in the present study penoxsulam was
only found in *S. plana* bivalves which were collected in August, in only one of the sampling stations (station B). Penoxsulam measured above its method quantification limit in both higher size classes (3+ and 4+ years old), with concentrations (whole organism) of 50 ng g\(^{-1}\) dw for the 3+ size class and of 124 ng g\(^{-1}\) dw for the 4+ size class. These results indicate higher accumulation by older organisms.

The herbicide terbuthylazine, together with atrazine (they both belong to the s-triazine chemical group), were the only pesticides found in macroalgae. Terbuthylazine, such as atrazine, was only found in August, thus suggesting a seasonal influence on its behaviour. A spatial gradient was also perceived, since this pesticide only measured above its method quantification limit in two sampling stations (station B: 108 ng g\(^{-1}\) dw, station C: 149 ng g\(^{-1}\) dw), suggesting that terbuthylazine mostly arrives to the south channel by River Pranto. This herbicide was also measured in *S. plana* tissues (only in August) collected in a single sampling station (station B) in both higher size classes (3+ and 4+ years old), with concentrations of 44 ng g\(^{-1}\) dw for the 3+ size class and of 88 ng g\(^{-1}\) dw for the 4+ size class. These results also indicate increment with age.

**Conclusions**

Quantified concentrations were determined mostly in August, in agreement with the pesticide application period. Among the six prospected pesticides, azoxystrobin presented the highest detection frequency, suggesting its wide use in the Lower Mondego. Notably, banned atrazine presented the second highest frequency, thus highlighting the need for legacy pesticides to be included in monitoring programmes in order to provide realistic pollutant assessment.

The bentazon concentrations in surface water determined in the present study were considerably higher than those reported for other countries, and they also exceeded
the European standard of 0.1 μg L⁻¹ for drinking water in all samples in both sampling periods.

The present study highlighted the limitations of the use of simple parameters, namely the Kow or the BCF, which are only based on substance specific properties. For instance, these parameters do not take into account pH and temperature, the presence of organic matter and mixtures of pollutants, or the metabolism of each organism. Hence, pesticide monitoring programmes are fundamental to identify realistic environmental exposures, as well as interpret living organism responses.

Animal species typically accumulated more pesticide residues than plant species. The gathered results highlighted the widespread interest of bivalves as biomonitor organisms of pesticides under realistic environmental conditions. It was possible to conclude that the age of *S. plana* determined clams’ susceptibility towards pesticide accumulation, with older animals being in general more vulnerable than younger ones. Older animals are larger and thus considered of commercial interest. Azoxystrobin was the dominant pesticide bioaccumulated in *S. plana*, and was measured in similar concentrations in individual organ (digestive gland) and the remaining tissues, suggesting that the use of whole body or digestive gland is equally valid when addressing the concentration of azoxystrobin in this species. Conversely, the other pesticides indicated higher metabolic rates by the digestive gland and rapid accumulation in other tissues. Thus, we recommend the use of whole-body 3+ or 4+ year-old *S. plana* for biomonitoring programmes of pesticides.

Findings of this study allowed the identification of an environmental hot spot located in the mouth of Esteiro dos Armazéns. The gathered results suggest that the pesticide residues were somehow trapped at that point. Even though further research is required to clarify this interaction, oil waste is a possibility. According to its webpage ([http://www.efp.pt/index.php/about-us/corporate-profile](http://www.efp.pt/index.php/about-us/corporate-profile), accessed Nov
the processing plant located upstream started to produce fine and pure squalene and squalane, and shark and cod liver oils in 2010, as well as products of vegetable origin by producing olive squalene, olive squalane, and grape seed oil, among others, in 2014.

Even though the present study does not address this item, the presence of pesticides in Ulva spp., G. gracilis and S. plana may not only cause adverse effects on the aquatic organisms themselves, but also pose a potential human health risk, since are all edible species and considered of economic interest (e.g., \( \lambda \)-cyhalothrin exceeded its maximum MRL over 10 times in \( S. \) plana). The risk is further increased as atrazine is regarded as a potential endocrine disruptor (Kucka et al., 2012). Acknowledging these concerns, developing and establishing allowable pesticide tolerance values is recommended, namely MRLs for edible seaweeds, fish, and crustaceans and molluscs (shellfish).
Chapter III – Azoxystrobin effects at the subcellular level


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5.559 IF_{2014} (6 citations (all), web of knowledge), Q1_{2014} Environmental sciences


doi 10.1016/j.chemosphere.2015.03.011
3.340 IF_{2014} (0 citations, web of knowledge), Q1_{2014} Environmental chemistry
THE CRAB CACINUS MAENAS AS A SUITABLE EXPERIMENTAL MODEL IN ECOTOXICOLOGY

This study aims to review the use of the crab *C. maenas* as an experimental test organism in aquatic ecotoxicology and to present an ecologically relevant study, which was conducted to evaluate the biochemical and physiological responses of *C. maenas* to temperature and azoxystrobin. Since *C. maenas* is replaced by its congeneric species *Carcinus aestuarii* in the Mediterranean Sea, ecotoxicological research concerning this closely related species was also included in the review. The two species are mainly distinguishable by morphological traits such as the shape of the pleopods in males, the shape of the frontal area between the eyes, and the carapace width to length ratio (Behrens Yamada and Hauck, 2001). This review describes strategies for capturing the crabs and their maintenance in laboratory, and discusses laboratory toxicity testing and *in vitro* approaches. Then, field studies using *C. maenas* follow, as well as the application of mesocosm experimental designs and general indices. Finally, the review aims to draw researchers' attention to the precautions which should be taken in future ecotoxicology studies using *C. maenas* as a test organism, so as to achieve reliable results. The ultimate purpose of the review is to provide valuable information for selecting and using appropriate bioassays to assess toxicity and biomonitor aquatic environmental health, from the molecular to the behavioural levels of the biological organisation of *Carcinus* sp.
Abstract

Aquatic ecotoxicology broadly focuses on how aquatic organisms interact with pollutants in their environment in order to determine environmental hazard and potential risks to human health. Research has produced increasing evidence on the pivotal role of aquatic invertebrates in the assessment of the impact of pollutants on the environment. Its potential use to replace fish bioassays, which offers ethical advantages, has already been widely studied. Nevertheless, the selection of adequate invertebrate experimental models, appropriate experimental designs and bioassays, as well as the control of potential confounding factors in toxicity testing are of major importance to obtain scientifically valid results. Therefore, the present project reviews more than four decades of published research papers in which the Green crab Carcinus maenas was used as an experimental test organism. In general, the surveyed literature indicates that Carcinus sp. (C. maenas and Carcinus aestuarii) is sensitive to a wide range of aquatic pollutants and that its biological responses are linked to exposure concentrations or doses. Current scientific knowledge regarding the biology and ecology of Carcinus sp. and the extensive studies on ecotoxicology found for the present review recognise the Green crab as a reliable marine model for routine testing in ecotoxicology research and environmental quality assessment, especially in what concerns the application of the biomarker approach. Data gathered provide valuable information for the selection of adequate and trustworthy bioassays to be used in Carcinus sp. toxicity testing. Since the final expression of high quality testing is a reliable outcome, the present review recommends gender, size and morphotype separation in Carcinus sp. experimental designs and data evaluation. Moreover, the organisms’ nutritional status should be taken into account, especially in long-term studies. Studies should also consider the crabs’ resilience when facing historical and concurrent
contamination. Finally, experimental temperature and salinity should be harmonised so as to obtain reliable comparisons between different studies. Concerning future research areas, data gathered in the present review reveals that *in vitro* assays derived from *Carcinus* sp. are still lacking. Also, a complete *Carcinus* sp. genome-sequencing programme will be essential for cutting-edge research.

***

**Laboratory studies**

Capture and maintenance strategies

Depending on whether the sampling site is intertidal or subtidal, crabs can be collected by hand, using traps (e.g., cages or nets), or by means of a beamtrawl, respectively. During transport to the laboratory, *C. maenas* can be placed in cool boxes with no water, since crabs can survive for at least five days out of water (Darbyson, 2006) and their ammonia excretion rate is strongly reduced during emersion periods (Durand and Regnault, 1998; Durand et al., 1999). This crab is perfectly able to regulate internal ammonia levels during prolonged emersion periods (up to 24 h) by means of an ammonia-detoxifying mechanism present in the muscle (Durand and Regnault, 1998; Durand et al., 1999). However, desiccation should be prevented by, e.g., joining damp seaweeds (Cripps et al., 2013) or damp absorbent paper (Dissanayake et al., 2011). Then, once at the laboratory, it should be taken into account that, upon reimmersion, crabs release large amounts of ammonia within a few minutes (Durand and Regnault, 1998). The ASTM E729 (2002) standard guide recommends acclimation in a constant-temperature room (22°C) with illumination programmed to a 16-h light and 8-h dark photoperiod for 14 days. Although this guideline considers a longer acclimation period as an improvement, almost all reviewed studies used an inferior period (Table 11). Most
studies carried out 7-d acclimations, thus hinting at possible unreported inconveniences of longer acclimation periods. For instance, authors' personal observations identified the beginning of the moult at 22°C 7-8 days after the organisms were caught from wild populations in winter, even when respecting a progressively temperature transition period.

Table 11 Acclimation conditions for Carcinus sp.

<table>
<thead>
<tr>
<th>No. of days</th>
<th>T (°C)</th>
<th>Salinity</th>
<th>Photoperiod (L: light, D: dark)</th>
<th>Seawater origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-24h</td>
<td>15</td>
<td>25-35</td>
<td>natural</td>
<td>natural (filtered)</td>
<td>Hagger et al., 2009</td>
</tr>
<tr>
<td>48h</td>
<td>15</td>
<td>34</td>
<td>natural (filtered, 10 µm)</td>
<td>natural (filtered)</td>
<td>Dissanayake et al. 2009a,b, 2009</td>
</tr>
<tr>
<td>48h-96h</td>
<td>15</td>
<td>30</td>
<td>natural (filtered)</td>
<td>natural (filtered)</td>
<td>Martin-Díaz et al., 2005</td>
</tr>
<tr>
<td>1</td>
<td>15</td>
<td>16-23</td>
<td>-</td>
<td>natural (filtered)</td>
<td>Pedersen et al., 1998</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>17.5, 35</td>
<td>12 h L:12 h D</td>
<td>-</td>
<td>Brown et al., 2004</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>Portmann, 1968</td>
</tr>
<tr>
<td>≥3</td>
<td>15</td>
<td>14-15</td>
<td>12 h L:12 h D</td>
<td>natural (filtered)</td>
<td>Rasmussen et al., 1995</td>
</tr>
<tr>
<td>3-7</td>
<td>13</td>
<td>14-15</td>
<td>natural</td>
<td>natural</td>
<td>Fehsenfeld et al., 2011</td>
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<tr>
<td>4</td>
<td>12</td>
<td></td>
<td>-</td>
<td>-</td>
<td>Gowland et al., 2002</td>
</tr>
<tr>
<td>7</td>
<td>17</td>
<td>33.8</td>
<td>12 h L:12 h D</td>
<td>natural (filtered)</td>
<td>Aguirre-Martínez et al., 2001a,b,c</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>34</td>
<td>12 h L:12 h D</td>
<td>natural (filtered)</td>
<td>Bamber and Depledge, 1997a</td>
</tr>
<tr>
<td>7</td>
<td>5, 15, 25, 35</td>
<td>12 h L:12 h D</td>
<td>natural</td>
<td>natural (filtered)</td>
<td>Camps et al., 2004</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>20</td>
<td>12 h L:12 h D</td>
<td>-</td>
<td>Dam et al., 2008</td>
</tr>
<tr>
<td>7</td>
<td>20</td>
<td>-</td>
<td>16 h L:8 h D</td>
<td>-</td>
<td>Elumalai et al., 2002</td>
</tr>
<tr>
<td>7</td>
<td>21</td>
<td>-</td>
<td>16 h L:8 h D</td>
<td>-</td>
<td>Elumalai et al., 2005</td>
</tr>
<tr>
<td>7</td>
<td>17.2</td>
<td>36.9</td>
<td>-</td>
<td>-</td>
<td>Ghedira et al., 2009</td>
</tr>
<tr>
<td>7</td>
<td>17.2</td>
<td>37</td>
<td>-</td>
<td>-</td>
<td>Ghedira et al., 2011</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>35</td>
<td>12 h L:12 h D</td>
<td>natural (filtered)</td>
<td>Lundebye and Depledge, 1998a,b</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>34-36</td>
<td>-</td>
<td>Lye et al., 2008</td>
<td></td>
</tr>
<tr>
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<td>15</td>
<td>34</td>
<td>natural (filtered)</td>
<td>natural (filtered)</td>
<td>Lundebye et al., 1997</td>
</tr>
<tr>
<td>7</td>
<td>16</td>
<td>15</td>
<td>14 h L:10 h D</td>
<td>natural (filtered)</td>
<td>Mesquita et al., 2011</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>several</td>
<td>12 h L:12 h D</td>
<td>reconstituted (Tropic Marin Nea)</td>
<td>Rainbow and Black, 2002</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>Reitz et al., 2003</td>
</tr>
<tr>
<td>7</td>
<td>17</td>
<td>35</td>
<td>-</td>
<td>natural</td>
<td>Ricciardi et al., 2008</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>15</td>
<td>-</td>
<td>natural (filtered)</td>
<td>Rodrigues et al., 2013a</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>Weeks et al., 1993</td>
</tr>
<tr>
<td>≥7</td>
<td>21</td>
<td>-</td>
<td>16 h L:8 h D</td>
<td>natural (filtered)</td>
<td>Elumalai et al., 2007</td>
</tr>
<tr>
<td>≥7</td>
<td>14</td>
<td>10</td>
<td>-</td>
<td>reconstituted (Marine Salt)</td>
<td>Fehsenfeld and Weihrauch, 2013</td>
</tr>
<tr>
<td>≥7</td>
<td>-</td>
<td>12</td>
<td>-</td>
<td>-</td>
<td>Skaggs and Henry, 2002</td>
</tr>
<tr>
<td>≥7</td>
<td>21</td>
<td>700 mOsm</td>
<td>-</td>
<td>-</td>
<td>Thurberg et al., 1973</td>
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<tr>
<td>7-8</td>
<td>15.5</td>
<td>400 mOsm</td>
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<td>Bjerregaard and Vislo, 1985</td>
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<td>natural (filtered, 1 µm)</td>
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<td>Cripps et al., 2013</td>
</tr>
<tr>
<td>7-10</td>
<td>20</td>
<td>33</td>
<td>14 h L:10 h D</td>
<td>reconstituted</td>
<td>Fossi et al., 1998</td>
</tr>
<tr>
<td>10</td>
<td>18</td>
<td>33</td>
<td>-</td>
<td>reconstituted</td>
<td>Fossi et al., 2000</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Morales-Caselles et al., 2008a,b</td>
</tr>
<tr>
<td>14</td>
<td>15</td>
<td>34</td>
<td>12 h L:12 h D</td>
<td>-</td>
<td>Dissanayake et al., 2010</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Martin-Díaz et al., 2004a</td>
</tr>
<tr>
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<td>15</td>
<td>33.8</td>
<td>-</td>
<td>-</td>
<td>Martin-Díaz et al., 2007</td>
</tr>
<tr>
<td>14</td>
<td>11</td>
<td>34</td>
<td>natural (filtered) sand filter</td>
<td>natural (filtered)</td>
<td>Sundt et al., 2006</td>
</tr>
<tr>
<td>≥14</td>
<td>11</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Hammer et al., 2012</td>
</tr>
<tr>
<td>21</td>
<td>12</td>
<td>10, 35</td>
<td>8 h L:16 h D</td>
<td>-</td>
<td>Lawson et al., 1995</td>
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<tr>
<td>21</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>natural (filtered)</td>
<td>Vedi and Depledge, 1995</td>
</tr>
<tr>
<td>27</td>
<td>15</td>
<td>14</td>
<td>16 h L:8 h D</td>
<td>natural (filtered)</td>
<td>Rodrigues et al., 2013b</td>
</tr>
<tr>
<td>21-28</td>
<td>15</td>
<td>10</td>
<td>natural</td>
<td>natural</td>
<td>Hansen et al., 1992a, 1992b</td>
</tr>
<tr>
<td>several</td>
<td>33</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Boitel and Truchot, 1989</td>
</tr>
</tbody>
</table>

* when not applied, more information is placed next to the value. T, temperature
During acclimation, the stock of organisms should be maintained in aerated recirculating aquatic systems composed by tanks and appropriate life support systems, and the vibration of tanks should be minimised (e.g., placing XPS extruded polystyrene pieces under the tanks). Each tank should be filled with natural seawater collected at an uncontaminated site, or with reconstituted water; and salinity should be progressively adjusted to 17 (ASTM E729, 2002). Some of the reviewed scientific studies reported filtration of natural seawater, even though the size of the mesh used was not always specified (Table 11). Reconstituted water may be prepared using a commercially obtained sea salt, e.g., tropic marin salt; or using laboratory reagent grade chemicals (Table 3 of ASTM E729 (2002)). In both cases, high-quality water should be used. Additionally, the importance of using a reproducible medium was reported by Rainbow and Black (2002) as crucial for toxicity tests with metal exposure. Since areas of shade are known to induce resting behaviour and prevent visual disturbance on crabs, shelters of PVC pipe or other materials should be supplied as environmental enrichment to each tank, as performed by Krång and Ekerholm (2006); and floating bio-balls have the positive effect of a stroller where the crabs hold upside down and move around the tank due to the water flow (authors' personal observation). Moreover, Matozzo et al. (2011a,b) specified as environmental enrichment the use of sand at the bottom of tanks. Finally, in order to avoid aggressive behaviours, crabs must be allocated on the basis of maturity and sex.

Toxicity testing and biomarkers

Pollutants enter the crab from water, sediment or food via the gills or the stomach, and accumulate in the hepatopancreas (Hp, also called midgut gland or digestive gland) via haemolymph (Hm) (Brouwer and Lee, 2007). The crustaceans' Hp performs many of the functions associated with the vertebrates' liver, pancreas and
small intestine, such as food absorption and transport, secretion of digestive enzymes and storage of lipids, glycogen, and a number of minerals (Brouwer and Lee, 2007; Felgenhauer, 1992). The measurement of crab responses (endpoints) to pollutants allows for the establishment of dose-response relationships, which form the basis of toxicity testing. However, in aquatic ecotoxicology, the dose is often replaced by concentration due to the difficulty to administer oral or injection doses. Dose- or concentration- response relationships can be obtained from laboratory and/or field studies.

EC$_{50}$, LC$_{50}$ and LT$_{50}$ data

In laboratory, toxicity is usually measured by standardised short-term tests, e.g., OECD 203 (1992) or US-EPA (1996), with mortality, immobility or growth inhibition the most common endpoints. These studies may generate further quantitative information, thus enabling the establishment of traditional ecotoxicological data, mostly expressed as the median effective or lethal concentration (EC$_{50}$ or LC$_{50}$). However, current literature on Carcinus sp. shows that EC$_{50}$ and LC$_{50}$ data are scarce, for only four EC$_{50}$ results were found in this literature survey which were determined from lysosomal membrane stability (LMS) responses after crabs’ exposure to the drugs caffeine, carbamazepine, ibuprofen and novobiocin (Table 12). Concerning short-term toxicity LC$_{50}$ data, the present review presents values for 21 different chemicals, metals mostly, with tributyltin oxide and silver nitrate the most harmful to crabs. Median lethal time (LT$_{50}$) values for copper(II) chloride, mercury(II) chloride and pyrene (PYR) are also presented in Table 12. Nevertheless, LC$_{50}$ values reported for Carcinus sp. are, in general, higher than those reported in the literature for other crustaceans, indicating the low sensitivity of this crab when assessing mortality as an endpoint (Elumalai et al., 2002, 2007).
Biomarker data

There is an increasing interest in determining potential long-term effects of continuous exposure to sublethal concentrations of pollutants usually found in natural environments, thus enabling a better extrapolation of ecotoxicology studies. Even though organisms may not die, they can have their functional activities compromised under sublethal concentrations, with potential negative consequences to the function of ecosystems and ecosystem services. This literature survey showed that, although used both in short- and long-term studies, the biomarker approach was mostly used in the assessment of long-term effects in *Carcinus* sp.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>CAS No.</th>
<th>Organism phase</th>
<th>Endpoint</th>
<th>Time of exposure: EC₅₀</th>
<th>Time of exposure: LC₅₀ (95% CL)</th>
<th>Concentration: LT₅₀</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>1,2,3,4,5,6-hexachlorocyclohexane</td>
<td>6087-31-1</td>
<td>-</td>
<td>-</td>
<td>48h: 100</td>
<td>-</td>
<td>-</td>
<td>ETOX database, 2014</td>
</tr>
<tr>
<td>4-nonylphenol</td>
<td>104-40-5</td>
<td>adult ♂</td>
<td>-</td>
<td>96h: 6.49 (4.76-8.49)</td>
<td>-</td>
<td>-</td>
<td>Ricciardi et al., 2008</td>
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<tr>
<td>6-chloro-N,N-diethyl-1,3,5-triazin-2,4-di-amine</td>
<td>122-34-9</td>
<td>-</td>
<td>-</td>
<td>48h: 100</td>
<td>-</td>
<td>-</td>
<td>ETOX database, 2014</td>
</tr>
<tr>
<td>antimony trichloride-dissolved after acidification</td>
<td>10025-91-9</td>
<td>zoeal</td>
<td>-</td>
<td>96h: 1-10</td>
<td>-</td>
<td>-</td>
<td>Amiard, 1976</td>
</tr>
<tr>
<td>antimony trichloride-dissolved after acidification</td>
<td>10025-91-9</td>
<td>adult</td>
<td>-</td>
<td>96h: 50</td>
<td>-</td>
<td>-</td>
<td>Amiard, 1976</td>
</tr>
<tr>
<td>antimony trichloride-oxaphenanthrene</td>
<td>10025-91-9</td>
<td>adult</td>
<td>-</td>
<td>96h: 1000</td>
<td>-</td>
<td>-</td>
<td>Amiard, 1976</td>
</tr>
<tr>
<td>cadmium chloride monohydrate</td>
<td>35657-65-2</td>
<td>juvenile ♂♀</td>
<td>-</td>
<td>48h: 27.92 (21.19-37.58)</td>
<td>-</td>
<td>-</td>
<td>Moreira et al., 2006</td>
</tr>
<tr>
<td>cadmium chloride monohydrate</td>
<td>35657-65-2</td>
<td>juvenile ♂♀</td>
<td>-</td>
<td>96h: 13.97 (9.73-18.51)</td>
<td>-</td>
<td>-</td>
<td>Moreira et al., 2006</td>
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<tr>
<td>caffeine</td>
<td>58-08-2</td>
<td>adult ♂</td>
<td>LMS: 26d: 19.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>carbamazepine</td>
<td>298-68-4</td>
<td>adult ♂</td>
<td>LMS: 26d: 0.32</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Aguirre-Martinez et al., 2013a</td>
</tr>
<tr>
<td>cobalt(Ⅱ) chloride</td>
<td>7646-79-9</td>
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<td>-</td>
<td>96h: 50</td>
<td>-</td>
<td>-</td>
<td>Amiard, 1976</td>
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<td>7646-79-9</td>
<td>adult</td>
<td>-</td>
<td>96h: 500-1000</td>
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<td>Amiard, 1976</td>
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<td>copper(Ⅱ) chloride</td>
<td>7447-39-4</td>
<td>adult ♂</td>
<td>-</td>
<td>-</td>
<td>1 mg L⁻¹ : 12</td>
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<td>Botil and Truchet, 1989</td>
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<td>-</td>
<td>2 mg L⁻¹ : 15</td>
<td>-</td>
<td>Botil and Truchet, 1989</td>
</tr>
<tr>
<td>copper(Ⅱ) sulfate</td>
<td>7758-96-7</td>
<td>adult</td>
<td>-</td>
<td>96h: 51.8</td>
<td>-</td>
<td>-</td>
<td>Elumalai et al., 2002</td>
</tr>
<tr>
<td>copper(Ⅱ) sulfate</td>
<td>7758-96-7</td>
<td>-</td>
<td>-</td>
<td>48h: 100</td>
<td>-</td>
<td>-</td>
<td>Portmann, 1968</td>
</tr>
<tr>
<td>copper(Ⅱ) sulfate + sodium dichromate</td>
<td>7921-01-3</td>
<td>adult</td>
<td>-</td>
<td>96h: 15.0 Cu + 43.6 Cr</td>
<td>-</td>
<td>-</td>
<td>Elumalai et al., 2002</td>
</tr>
<tr>
<td>dibutylphthalate</td>
<td>62-56-1</td>
<td>adult</td>
<td>LMS: 26d: 0.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Aguirre-Martinez et al., 2013a</td>
</tr>
<tr>
<td>mercury(Ⅱ) chloride</td>
<td>7487-94-7</td>
<td>adult</td>
<td>-</td>
<td>48h: 1.5</td>
<td>-</td>
<td>-</td>
<td>Portmann, 1968</td>
</tr>
<tr>
<td>mercury(Ⅱ) chloride</td>
<td>7487-94-7</td>
<td>adult</td>
<td>-</td>
<td>250 µg L⁻¹ : 7</td>
<td>-</td>
<td>-</td>
<td>Bjerregaard and Vislie, 1985</td>
</tr>
<tr>
<td>mercury(Ⅱ) chloride</td>
<td>7487-94-7</td>
<td>adult</td>
<td>-</td>
<td>500 µg L⁻¹ : 15</td>
<td>-</td>
<td>-</td>
<td>Bjerregaard and Vislie, 1985</td>
</tr>
<tr>
<td>mercury(Ⅱ) chloride</td>
<td>7487-94-7</td>
<td>adult</td>
<td>-</td>
<td>1 mg L⁻¹ : 3</td>
<td>-</td>
<td>-</td>
<td>Bjerregaard and Vislie, 1985</td>
</tr>
<tr>
<td>mercury(Ⅱ) chloride</td>
<td>7487-94-7</td>
<td>adult</td>
<td>-</td>
<td>10 mg L⁻¹ : 1</td>
<td>-</td>
<td>-</td>
<td>Bjerregaard and Vislie, 1985</td>
</tr>
<tr>
<td>nickel(Ⅱ) chloride</td>
<td>7788-81-4</td>
<td>adult</td>
<td>-</td>
<td>48h : 1000</td>
<td>-</td>
<td>-</td>
<td>Portmann, 1968</td>
</tr>
<tr>
<td>novobiocin</td>
<td>303-81-1</td>
<td>adult</td>
<td>LMS: 26d: 2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Aguirre-Martinez et al., 2013a</td>
</tr>
<tr>
<td>phenol</td>
<td>106-20-5</td>
<td>-</td>
<td>-</td>
<td>48h: 75</td>
<td>-</td>
<td>-</td>
<td>Portmann, 1968</td>
</tr>
<tr>
<td>pyrene (via injection each fifth day)</td>
<td>129-00-0</td>
<td>green adult</td>
<td>-</td>
<td>-</td>
<td>6 µg g⁻¹ ww: 34.1</td>
<td>-</td>
<td>Dam et al., 2006</td>
</tr>
<tr>
<td>pyrene (via injection each fifth day)</td>
<td>129-00-0</td>
<td>red adult</td>
<td>-</td>
<td>-</td>
<td>6 µg g⁻¹ ww: 24.4</td>
<td>-</td>
<td>Dam et al., 2006</td>
</tr>
<tr>
<td>sodium dichromate (hexavalent chromium)</td>
<td>10588-01-9</td>
<td>adult</td>
<td>-</td>
<td>96h: 49.8</td>
<td>-</td>
<td>-</td>
<td>Elumalai et al., 2002</td>
</tr>
<tr>
<td>silver nitrate</td>
<td>7761-88-2</td>
<td>zoeal</td>
<td>-</td>
<td>96h: 0.01-0.1</td>
<td>-</td>
<td>-</td>
<td>Amiard, 1976</td>
</tr>
<tr>
<td>silver nitrate</td>
<td>7761-88-2</td>
<td>adult</td>
<td>-</td>
<td>96h: 1-2</td>
<td>-</td>
<td>-</td>
<td>Amiard, 1976</td>
</tr>
<tr>
<td>strontium chloride</td>
<td>10025-70-4</td>
<td>zoeal</td>
<td>-</td>
<td>96h: 10-100</td>
<td>-</td>
<td>-</td>
<td>Amiard, 1976</td>
</tr>
<tr>
<td>strontium chloride</td>
<td>10025-70-4</td>
<td>adult</td>
<td>-</td>
<td>96h: 1000</td>
<td>-</td>
<td>-</td>
<td>Amiard, 1976</td>
</tr>
<tr>
<td>tributyltin oxide</td>
<td>56-35-9</td>
<td>-</td>
<td>-</td>
<td>48h: 0.11</td>
<td>-</td>
<td>-</td>
<td>ETOX database, 2014</td>
</tr>
<tr>
<td>tributyltin oxide</td>
<td>56-35-9</td>
<td>-</td>
<td>-</td>
<td>96h: 0.01</td>
<td>-</td>
<td>-</td>
<td>ETOX database, 2014</td>
</tr>
<tr>
<td>zinc sulfate</td>
<td>7733-02-0</td>
<td>adult</td>
<td>-</td>
<td>96h: 14.88</td>
<td>-</td>
<td>-</td>
<td>Elumalai et al., 2007</td>
</tr>
<tr>
<td>zinc sulfate</td>
<td>7733-02-0</td>
<td>-</td>
<td>-</td>
<td>48h: 20</td>
<td>-</td>
<td>-</td>
<td>Portmann, 1968</td>
</tr>
</tbody>
</table>
assays. In general, and even though several definitions are possible, a biological response measured by endpoints from molecular to behavioural levels, providing exposure and/or effect evidence of increasing concentrations of a specific pollutant or a group of pollutants is called a biomarker. Thus, biomarkers characterize the bioavailable fraction of environmental pollutants and are, therefore, currently considered potential tools to be applied in ERA by using Carcinus sp. as a test organism (Fillmann et al., 2002; Galloway, 2006). Moreover, biomarkers are rapid and easy to use, and are, in general, considered a cost-effective approach for identifying the toxic effects of pollutants on biota (e.g., Brown et al., 2004; Galloway et al., 2002, 2004a). However, in order to be routinely applied in toxicity testing using Carcinus sp., this approach must previously be vastly tested and validated. Therefore, a summary of the data of the biomarker approach by response criteria gathered from the surveyed literature is presented below. The effects of metals on C. maenas were the most commonly reported, and copper (Cu) was the most studied metal. Nevertheless, pharmaceutical drugs, endocrine disruptors, PAHs, PCBs, pesticides and other metals were also found in the reviewed studies. The response criteria used varied between molecular, biochemical, cellular, physiological, histomorphological, and behavioural endpoints, representing the various levels of biological organisation.

Molecular endpoints

Genotoxicity usually encompasses all DNA damaging effects representing molecular endpoints. Assays in aquatic invertebrates were reviewed by Dixon et al. (2002) and Galloway et al. (2010). The genotoxicity of drugs, PAHs (benzopyrene (BP)), PCBs (aroclor 1260), and methyl mercury (MeHg) was tested on Carcinus sp. using the alkaline unwinding and the alkaline precipitation assays by Fossi et al. (1996, 2000) and by Aguirre-Martínez et al. (2013b,c), respectively (Table 13). Both
methods can assess DNA damage by measuring its strand breaks. However, DNA damage may also be determined at chromosome level by performing the micronucleus formation (MN) assay, which can measure both chromosome loss and chromosome breakage. Accordingly, Fossi et al. (2000) employed this method to signal exposure to BP using the Hm of male C. aequarii. From Table 13 it is possible to conclude that the assessment of DNA damage through the alkaline precipitation assay seems to be a suitable molecular biomarker to assess effects of environmental concentrations of drugs. However, results were found to be tissue dependent. Concerning the study of BP carried out by Fossi et al. (2000), even though showing significant responses, the results failed to obtain a concentration-response relationship. One of the possible reasons for the absence of such relationship may have been the choice of the response criteria, since BP is classified as carcinogenic (group 1; IARC, 2010) and the most common mechanism of carcinogenesis induced by PAHs is the formation of DNA adducts (Beland and Poirier, 1994; Muñoz and Albores, 2011). However, Fossi et al. (2000) studied both DNA strand breaks and the formation of micronucleus. Therefore, further research is needed on the quantitative correlation between the level of BP exposure and the molecular responses of crabs.

### Table 13

The use of Carcinus sp. to assess DNA damage in laboratory toxicity studies. Successful outcomes are highlighted.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Exposure chemical</th>
<th>Sensitivity (significantly lower dose or concentration)</th>
<th>Dose- or concentration-response relationship</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>alkaline precipitation</td>
<td>drugs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>caffeine</td>
<td>0.1 - 50 µg L⁻¹</td>
<td>28d (adult) : 0.1 (gall***), 15 (Hpm***, Mus***)</td>
<td>= (Hpm**, gall**, Mus**, Gon**) neg (Gon**)</td>
<td>Agum-Martinez et al., 2013b</td>
</tr>
<tr>
<td>carbamazepine</td>
<td>0.1 - 50 µg L⁻¹</td>
<td>28d (adult) : 0.1 (gall***), 15 (Hpm***, Mus***)</td>
<td>= (Hpm**, gall**, Mus**, Gon**) neg (Gon**)</td>
<td>Agum-Martinez et al., 2013b</td>
</tr>
<tr>
<td>ibuprofen</td>
<td>0.1 - 50 µg L⁻¹</td>
<td>28d (adult) : 0.1 (gall***), 5 (gall***), 50 (Hpm***)</td>
<td>= (Hpm**, gall**, Mus**, Gon***)</td>
<td>Agum-Martinez et al., 2013b</td>
</tr>
<tr>
<td>novobiocin</td>
<td>0.1 - 50 µg L⁻¹</td>
<td>28d (adult) : 0.1 (Gon***), 10 (gall***), 50 (Hpm***)</td>
<td>= (Hpm**, gall**, Mus**, Gon***)</td>
<td>Agum-Martinez et al., 2013b</td>
</tr>
<tr>
<td>alkaline unwinding</td>
<td>PAHs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP</td>
<td>1, 10 µg, via injection</td>
<td>72h adult : 1 (Hp)</td>
<td>not reported</td>
<td>Fossi et al., 1996</td>
</tr>
<tr>
<td>BP</td>
<td>1 - 1000 µg L⁻¹</td>
<td>10d adult : 1 (Hp)</td>
<td>no significant relationship</td>
<td>Fossi et al., 2000</td>
</tr>
<tr>
<td>PCBs</td>
<td>arcole 1260</td>
<td></td>
<td></td>
<td>Fossi et al., 1996</td>
</tr>
<tr>
<td>BP</td>
<td>10, 100 µg, via injection</td>
<td>72h adult : 10 (Hp)</td>
<td>not reported</td>
<td>Fossi et al., 1996</td>
</tr>
<tr>
<td>organometals</td>
<td>Methyl</td>
<td></td>
<td></td>
<td>Fossi et al., 1996</td>
</tr>
<tr>
<td>BP</td>
<td>1 - 1000 µg L⁻¹</td>
<td>10d adult : 1000 (Hpm**)</td>
<td>no significant relationship</td>
<td>Fossi et al., 2000</td>
</tr>
</tbody>
</table>


Significant level: *P*<0.001, **P*<0.01, ***P*<0.05
Biochemical endpoints

Crabs can metabolise some organic pollutants in phase I metabolism through oxidation, reduction or hydrolysis reactions promoted by several enzymes. Usually, through this process, apolar molecules are made more polar and more water soluble, thus becoming excretable. For instance, microsomal monooxygenase enzymes (also called mixed-function oxidase (MFO) enzymes), which function through the hemoprotein cytochrome P450 (CYP), can metabolise toxic molecules at the expense of molecular oxygen. The superfamily of CYP enzymes was reviewed by Snyder (2000) and by Rewitz et al. (2006) in aquatic invertebrates, and especially in crustaceans by James and Boyle (1998). Moreover, concerning C. maenas, Solé and Livingstone (2005) found high levels of total CYP enzymes in Hp (223 pmol mg⁻¹). Also, among epidermis, gills, Hp, muscle and gonads, the Hp was considered by Dam et al. (2008) as the major site of adult male C. maenas's CYPs gene expression. Both studies allowed us to conclude that the Hp plays a key role as a target detoxification organ in Carcinus sp. Furthermore, subfamilies CYP2C (stands for family 2, subfamily C) and CYP3A are the most abundant in crustaceans' Hp microsomes (James and Boyle, 1998). The MFO system also incorporates cytochrome b5 (measured by NADH-ferricyanide reductase (NADH-FR) activity) and NADPH cytochrome (measured by NADPH cytochrome c reductase (NADPH-CcR) activity). NADH cytochrome c reductase (NADH-CcR) activity can also be measured as an endpoint since it is catalysed by both cytochrome b5 reductase and cytochrome b5. A high level of NADH-FR activity was found in the Hp of C. maenas (436 nmol min⁻¹ mg⁻¹ protein) by Solé and Livingstone (2005). However, low NADPH-CcR and NADH-CcR activities were reported in the same study, with the values of 4.0 and 22.5 nmol min⁻¹ mg⁻¹ protein, respectively. Table 14 reports MFO system toxicity studies performed with Carcinus sp. Table 14 does not provide clear evidence of benzopyrene monoxygenase (BPMO) and NADH- and NADPH-
dependent CYP activities as suitable endpoints of *Carcinus* sp. toxicity testing. Moreover, CYP2 gene expression analysis, also called CYP2 genomics, appears to be sensitive for future research with PAHs. However, concentration-response relationships have not yet been achieved. Table 14 also shows the induction of ethoxyresorufin-O-deethylase (EROD) and benzo(a)pyrene hydroxylase (BPH), both characteristic of vertebrate CYP1A. Nevertheless, CYP1A genes have not yet been found in any invertebrate genome. Thus, although EROD seems to be a suitable endpoint to be used in toxicity testing, further studies are needed to clarify the role of this enzyme among invertebrates. Concerning BPH, the significantly lower concentration of BP which induced Hp and gills of male *C. maenas* was 1 mg L⁻¹. However, and even though BP concentrations in ambient estuarine and coastal waters are not well documented in literature, the environmental concentrations found in lakes and rivers were in the range of nanograms (e.g., Kabziński et al., 2002; Rhea et al., 2005; Wang et al., 2011; Zhang et al., 2012). Therefore, although a quantitative positive correlation between BP exposure level and crab responses was found by Fossi et al. (1998), BPH is not considered in the present review as an ecologically relevant endpoint for aquatic BP contamination. On the other hand, dibenzylfluorescein dealkylase (DBF), which was used to assess CYP3A4 responses, seems to have potential as a phase I biomarker of crabs' MFO when environmental concentrations of some drugs are studied.
### Table 14
The use of *Carcinus* sp. to assess the responses of the mixed-function oxidase system in laboratory toxicity studies. Successful outcomes are highlighted.

<table>
<thead>
<tr>
<th>Endpoint assay</th>
<th>Group</th>
<th>Name</th>
<th>Concentration range</th>
<th>Sensitivity (significantly lower dose or concentration)</th>
<th>Dose- or concentration-response relationship</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EROD</td>
<td>drugs</td>
<td>caffeine</td>
<td>0.1 - 50 μg L⁻¹</td>
<td>28d adult: &lt; 0.1 (Hp**),</td>
<td>+ (Hp**, Gen***)</td>
<td>Agüero-Martínez et al., 2013b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>carbamazepine</td>
<td>0.1 - 50 μg L⁻¹</td>
<td>28d adult: &lt; 0.1 (Mux**), 1 (Hp**), 50 (Hp***)</td>
<td>+ (Hp**)</td>
<td>Agüero-Martínez et al., 2013c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ibuprofen</td>
<td>0.1 - 50 μg L⁻¹</td>
<td>28d adult: &lt; 0.1 (Hp**),</td>
<td>+ (Hp**)</td>
<td>Agüero-Martínez et al., 2013c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>noreadoxin</td>
<td>0.1 - 50 μg L⁻¹</td>
<td>28d adult: &lt; 0.1 (Hp**, Mux**, Gen***)</td>
<td>+ (Hp**, Mux**, Gen***)</td>
<td>Agüero-Martínez et al., 2013c</td>
</tr>
<tr>
<td>PAHs</td>
<td>BP</td>
<td>1, 10 μg via injection</td>
<td>72h adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td></td>
<td>Fossi et al., 1996, 1998</td>
</tr>
<tr>
<td></td>
<td>BP</td>
<td>1 - 1000 μg L⁻¹</td>
<td>10d adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td>no significant relationship</td>
<td>Fossi et al., 1996, 2001</td>
</tr>
<tr>
<td>PCBs</td>
<td>arsor dur 120</td>
<td>10, 100 μg, via injection</td>
<td>72h adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td></td>
<td>Fossi et al., 1996, 1998</td>
</tr>
<tr>
<td>organometals</td>
<td>MeHg</td>
<td>1, 10 μg, via injection</td>
<td>72h adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td></td>
<td>Fossi et al., 1996, 1998</td>
</tr>
<tr>
<td>PAHs, PCBs, metals</td>
<td>sediments</td>
<td>-</td>
<td>28d adult: &lt; (PCBs (Hp**))</td>
<td>+ (Hp, PCBs**)</td>
<td></td>
<td>Martin-Chur et al., 2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sediments</td>
<td>-</td>
<td>28d all contaminated sediments**</td>
<td>+ (Hp, PAHs**, PCBs**, Cr, Ni**)</td>
<td>Monzó-Casselles et al., 2006b</td>
</tr>
<tr>
<td>BPMO</td>
<td>PAHs</td>
<td>BP</td>
<td>1, 10 μg via injection</td>
<td>72h 1 (Hp, gil)</td>
<td>not reported</td>
<td>Fossi et al., 1996</td>
</tr>
<tr>
<td></td>
<td>BP</td>
<td>1 - 1000 μg L⁻¹</td>
<td>10d adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td>+ (Hp**)</td>
<td>Fossi et al., 2003</td>
</tr>
<tr>
<td>PCBs</td>
<td>arsor dur 120</td>
<td>10, 100 μg, via injection</td>
<td>72h 100 (Hp)</td>
<td>not reported</td>
<td></td>
<td>Fossi et al., 1996</td>
</tr>
<tr>
<td>organometals</td>
<td>MeHg</td>
<td>1, 10 μg, via injection</td>
<td>72h adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td></td>
<td>Fossi et al., 1996</td>
</tr>
<tr>
<td></td>
<td>BP</td>
<td>1 - 1000 μg L⁻¹</td>
<td>10d adult: &lt; 0.1 (Hp**, gil**)</td>
<td>not reported</td>
<td>+ (Hp, gil)</td>
<td>Fossi et al., 1996</td>
</tr>
<tr>
<td>gene expression (CYP2)</td>
<td>drugs</td>
<td>clofibrate</td>
<td>0.8, 8 μg, via injection</td>
<td>96h adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td>Revert et al., 2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>phenobarbital</td>
<td>0.04, 0.4 mg, via injection</td>
<td>96h adult: &lt; 0.04 (Hp*)</td>
<td>upregulated</td>
<td>not reported</td>
</tr>
<tr>
<td>PAHs</td>
<td>BP</td>
<td>0.4, 4 mg, via injection</td>
<td>96h adult: &lt; 0.1 (Hp**)</td>
<td>upregulated</td>
<td>not reported</td>
<td>Revert et al., 2003</td>
</tr>
<tr>
<td></td>
<td>BP</td>
<td>0.12, 1.2 mg, via injection</td>
<td>96h adult: &lt; 0.12 (Hp**)</td>
<td>upregulated</td>
<td>not reported</td>
<td>Revert et al., 2003</td>
</tr>
<tr>
<td></td>
<td>BP</td>
<td>1.2 mg, via injection</td>
<td>96h adult: &lt; 1.2 (Hp**)</td>
<td>upregulated</td>
<td>not reported</td>
<td>Revert et al., 2003</td>
</tr>
<tr>
<td>gene expression (CYP3A)</td>
<td>drugs</td>
<td>phenobarbital</td>
<td>0.4 mg, via injection</td>
<td>96h adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td>Fossi et al., 2008</td>
</tr>
<tr>
<td></td>
<td>PAHs</td>
<td>BP</td>
<td>1.2 mg, via injection</td>
<td>96h adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td>Fossi et al., 2008</td>
</tr>
<tr>
<td></td>
<td>BP</td>
<td>1.2 mg, via injection</td>
<td>96h adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td></td>
<td>Fossi et al., 2008</td>
</tr>
<tr>
<td></td>
<td>PYR</td>
<td>1.2 mg, via injection</td>
<td>96h adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td></td>
<td>Fossi et al., 2008</td>
</tr>
<tr>
<td>gene expression (CYP3A)</td>
<td>drugs</td>
<td>phenobarbital</td>
<td>0.4 mg, via injection</td>
<td>96h adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td>Fossi et al., 2008</td>
</tr>
<tr>
<td></td>
<td>PAHs</td>
<td>BP</td>
<td>1.2 mg, via injection</td>
<td>96h adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td>Fossi et al., 2008</td>
</tr>
<tr>
<td></td>
<td>BP</td>
<td>1.2 mg, via injection</td>
<td>96h adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td></td>
<td>Fossi et al., 2008</td>
</tr>
<tr>
<td>gene expression (CYP450)</td>
<td>drugs</td>
<td>phenobarbital</td>
<td>0.4 mg, via injection</td>
<td>96h adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td>Fossi et al., 2008</td>
</tr>
<tr>
<td></td>
<td>PAHs</td>
<td>BP</td>
<td>1.2 mg, via injection</td>
<td>96h adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td>Fossi et al., 2008</td>
</tr>
<tr>
<td></td>
<td>BP</td>
<td>1.2 mg, via injection</td>
<td>96h adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td></td>
<td>Fossi et al., 2008</td>
</tr>
<tr>
<td>gene expression (CYP450)</td>
<td>drugs</td>
<td>phenobarbital</td>
<td>0.4 mg, via injection</td>
<td>96h adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td>Fossi et al., 2008</td>
</tr>
<tr>
<td></td>
<td>PAHs</td>
<td>BP</td>
<td>1.2 mg, via injection</td>
<td>96h adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td>Fossi et al., 2008</td>
</tr>
<tr>
<td></td>
<td>BP</td>
<td>1.2 mg, via injection</td>
<td>96h adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td></td>
<td>Fossi et al., 2008</td>
</tr>
<tr>
<td>NADH-CuR</td>
<td>PAHs</td>
<td>BP</td>
<td>1, 10 μg, via injection</td>
<td>72h adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td>Fossi et al., 1998</td>
</tr>
<tr>
<td></td>
<td>BP</td>
<td>1 - 1000 μg L⁻¹</td>
<td>10d adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td>no significant relationship</td>
<td>Fossi et al., 2000</td>
</tr>
<tr>
<td>organometals</td>
<td>MeHg</td>
<td>1, 10 μg, via injection</td>
<td>72h adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td></td>
<td>Fossi et al., 1998</td>
</tr>
<tr>
<td>organometals</td>
<td>BP</td>
<td>1 - 1000 μg L⁻¹</td>
<td>10d adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td>no significant relationship</td>
<td>Fossi et al., 2000</td>
</tr>
<tr>
<td>NADPH-CuR</td>
<td>PAHs</td>
<td>BP</td>
<td>1, 10 μg, via injection</td>
<td>72h adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td>Fossi et al., 1998</td>
</tr>
<tr>
<td></td>
<td>BP</td>
<td>1 - 1000 μg L⁻¹</td>
<td>10d adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td>no significant relationship</td>
<td>Fossi et al., 2000</td>
</tr>
<tr>
<td>organometals</td>
<td>MeHg</td>
<td>1, 10 μg, via injection</td>
<td>72h adult: &lt; 0.1 (Hp**)</td>
<td>not reported</td>
<td></td>
<td>Fossi et al., 1998</td>
</tr>
</tbody>
</table>

Significant level: *P*≤0.05, *P*≤0.01, *P*≤0.001

Phase I metabolism responses of *Carcinus* sp. were also attained by measuring esterase activities promoted by drugs, PAHs, PCBs, pesticides, organometals and metals (Table 15). Data gathered showed that acetylcholinesterase (AChE) catalysed enzymatic reactions were the most used to signal exposure to a wide
range of pollutants. This enzyme plays a pivotal role associated with neurotransmission, since it is responsible for hydrolysing the neurotransmitter acetylcholine into choline and acetic acid. Thus is a useful enzyme to assess neurotoxicity, e.g., promoted by organophosphate or carbamate pesticides. Other specific esterases found in this literature survey were butyrylcholinesterase (BChE) and carboxylesterase (CBE). The latter is capable of cleaving the carboxyl group of pollutants, namely organophosphate pesticides. In general, esterase activity seems to be a sensitive biomarker to assess responses to pesticides and metals when *Carcinus* sp. is used. Nevertheless, concentration-response relationships were mostly found when non-specific esterase activity was measured, possibly due to the fact that a clear differentiation between, e.g., AChE and BChE is only possible in the higher vertebrates (*Urich, 1990*). Table 15 indicates that Cr (chromium) and Cu exposures have occurred, and a concentration-response between both metals and non-specific esterases was attained. Yet, environmental concentrations of Cr and Cu in surface waters are in the range of micrograms (e.g., Baralkiewicz and Siepak, 1999; Samecka-Cymerman and Kempers, 2004; Vasilatos et al., 2008; Wang and Zang, 2014; Wilbers et al., 2014). Therefore, esterase activity is not considered an ecologically relevant endpoint to assess Cr and Cu contamination when gonad tissue of female *Carcinus* sp. is used. The same conclusion is reached with regard to AChE and fluoranthene when male crabs' muscle tissue is used, since environmental concentrations of fluoranthene are usually in the range of nanograms (e.g., Kabziński et al., 2002; Wang et al., 2011; Zhang et al., 2012). Conversely, environmental concentrations of mercury (Hg) and zinc (Zn) seem to induce male *C. maenas* eye cholinesterase (ChE) in a negative direct relation, being considered a suitable endpoint.
It is known that the organisms' phase I metabolism may produce ROS as by-products (Livingstone, 1991). As ROS are highly reactive molecules, they can damage cell components, including DNA, proteins and membranes, causing a phenomenon known as oxidative stress (reviewed by Lushchak (2011) in aquatic organisms). Cells possess a complex defence system to protect themselves from ROS, including non-enzymatic scavengers (e.g., reduced glutathione (GSH), vitamins C and E) and antioxidant enzymes (e.g., catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD)). Nevertheless, the activities of 6-phosphogluconate dehydrogenase (6PGDH), glutathione reductase (GR), and NADP+-dependent isocitrate dehydrogenase (IDH), as well as the total glutathione (TG) level, can also be measured to assess oxidative stress responses in ecotoxicity testing. 6PGDH and IDH act as antioxidant enzymes by supplying
NADPH to cytosol for further use by GR, and GR promotes the recycling of oxidised glutathione into GSH, maintaining its cellular level (Lee et al., 2002). Studies regarding pollutant ability to disturb the anti-oxidant system of Carcinus sp. are presented in Table 16. It is therefore possible to conclude that all the used endpoints (6PGDR, CAT, GPx, GR, IDH and TG) were, in general, sensitive to assess anti-oxidant responses of Carcinus sp. Moreover, significant concentration-response relationships were attained between GPx and drugs, GR and sediment contamination (Zn), and IDH and TG, both with PAHs (fluoranthene). Nevertheless, IDH and TG are not considered ecologically relevant endpoints to assess fluoranthene contamination when male Hp and muscle tissue are used, since environmental concentrations of fluoranthene are usually in the range of nanograms (e.g., Kabziński et al., 2002; Wang et al., 2011; Zhang et al., 2012).

Table 16 The use of Carcinus sp. to assess oxidative stress responses in laboratory toxicity studies. Successful outcomes are highlighted.

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Group</th>
<th>Exposure chemical</th>
<th>Concentration range</th>
<th>Sensitivity (significantly lower dose or concentration)</th>
<th>Dose-concentration response relationship</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>6PGDR</td>
<td>pesticides + metals</td>
<td>Dumbaranî (chloropyrrolophenyl)</td>
<td>3.12 µg L⁻¹</td>
<td>adult: 3.12 (Hp⁺⁺, gill⁺⁺)</td>
<td>-</td>
<td>Ghedira et al., 2011</td>
</tr>
<tr>
<td></td>
<td>pesticides + metals</td>
<td>Dumbaranî + Cd</td>
<td>3.12 + 500 µg L⁻¹</td>
<td>adult: 3.12 + 500 (Hp⁺⁺, gill⁺⁺)</td>
<td>-</td>
<td>Ghedira et al., 2011</td>
</tr>
<tr>
<td></td>
<td>pesticides + metals</td>
<td>Dumbaranî (chloropyrrolophenyl)</td>
<td>3.12 µg L⁻¹</td>
<td>adult: 3.12 (Hp⁺⁺, gill⁺⁺)</td>
<td>-</td>
<td>Ghedira et al., 2011</td>
</tr>
<tr>
<td></td>
<td>metals</td>
<td>Cd</td>
<td>500 µg L⁻¹</td>
<td>adult: 500 (Hp⁺⁺⁺)</td>
<td>-</td>
<td>Ghedira et al., 2011</td>
</tr>
<tr>
<td>CAT</td>
<td>pesticides + metals</td>
<td>Dumbaranî (chloropyrrolophenyl)</td>
<td>3.12 µg L⁻¹</td>
<td>adult: 3.12 + 500 (Hp⁺⁺, gill⁺⁺)</td>
<td>-</td>
<td>Ghedira et al., 2011</td>
</tr>
<tr>
<td></td>
<td>metals</td>
<td>Cd</td>
<td>500 µg L⁻¹</td>
<td>adult: 500 (Hp⁺⁺, gill⁺⁺)</td>
<td>-</td>
<td>Ghedira et al., 2011</td>
</tr>
<tr>
<td></td>
<td>pesticides + metals</td>
<td>Dumbaranî + Cd</td>
<td>3.12 + 500 µg L⁻¹</td>
<td>adult: 3.12 + 500 (Hp⁺⁺, gill⁺⁺)</td>
<td>-</td>
<td>Ghedira et al., 2011</td>
</tr>
<tr>
<td>GPx</td>
<td>drugs</td>
<td>caffeine</td>
<td>0.1 - 50 µg L⁻¹</td>
<td>adult: 5 (Hp⁺⁺⁺, gill⁺⁺⁺, Mus⁺⁺⁺, Gor⁺⁺⁺)</td>
<td>-</td>
<td>Ghedira et al., 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>carbamazepine</td>
<td>0.1 - 50 µg L⁻¹</td>
<td>adult: ≤ 1 (Gor⁺⁺⁺), 10 (Hp⁺⁺⁺, gill⁺⁺⁺, Mus⁺⁺⁺)</td>
<td>+ (Hp⁺⁺⁺, gill⁺⁺⁺, Gor⁺⁺⁺)</td>
<td>Aguirre-Martínez et al., 2013b</td>
</tr>
<tr>
<td></td>
<td>drugs</td>
<td>fluoxetine</td>
<td>0.5 - 750 µg L⁻¹</td>
<td>adult: no significant effect</td>
<td>not reported</td>
<td>Mosquita et al., 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>lornipirien</td>
<td>0.1 - 50 µg L⁻¹</td>
<td>adult: ≤ 0.1 (Gor⁺⁺⁺), 5 (Hp⁺⁺⁺, Mus⁺⁺⁺), 10 (Mus⁺⁺⁺)</td>
<td>+ (Hp⁺⁺⁺, Mus⁺⁺⁺, Gor⁺⁺⁺)</td>
<td>Aguirre-Martínez et al., 2013b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>novobiocin</td>
<td>0.1 - 50 µg L⁻¹</td>
<td>adult: ≤ 1 (Gor⁺⁺⁺), 10 (Mus⁺⁺⁺), 50 (Gor⁺⁺⁺)</td>
<td>+ (Hp⁺⁺⁺, Mus⁺⁺⁺, Gor⁺⁺⁺)</td>
<td>Aguirre-Martínez et al., 2013b</td>
</tr>
<tr>
<td>PAHs</td>
<td>sediments</td>
<td>fluoranthene</td>
<td>2.56 - 100 µg L⁻¹</td>
<td>adult: no significant effect</td>
<td>no significant relationship</td>
<td>Rodríguez et al., 2013a</td>
</tr>
<tr>
<td></td>
<td>sediments</td>
<td>-</td>
<td>28d</td>
<td>adult: Hp⁺⁺⁺, PCBs</td>
<td>no significant relationship</td>
<td>Martin-Díaz et al., 2007</td>
</tr>
<tr>
<td></td>
<td>pesticis</td>
<td>Dumbaranî (chloropyrrolophenyl)</td>
<td>3.12 µg L⁻¹</td>
<td>adult: no significant effect</td>
<td>-</td>
<td>Ghedira et al., 2011</td>
</tr>
<tr>
<td></td>
<td>metals</td>
<td>Cd</td>
<td>500 µg L⁻¹</td>
<td>adult: no significant effect</td>
<td>-</td>
<td>Ghedira et al., 2011</td>
</tr>
<tr>
<td>GR</td>
<td>pesticides + metals</td>
<td>Dumbaranî + Cd</td>
<td>3.12 + 500 µg L⁻¹</td>
<td>adult: 3.12 + 500 (Hp⁺⁺⁺)</td>
<td>-</td>
<td>Ghedira et al., 2011</td>
</tr>
<tr>
<td></td>
<td>PAHs</td>
<td>fluoranthene</td>
<td>2.56 - 100 µg L⁻¹</td>
<td>adult: ≤ 40 (Hp⁺⁺⁺)</td>
<td>not reported</td>
<td>Ghedira et al., 2011</td>
</tr>
<tr>
<td></td>
<td>sediments</td>
<td>-</td>
<td>28d</td>
<td>adult: no significant effect</td>
<td>no significant relationship</td>
<td>Rodríguez et al., 2013a</td>
</tr>
<tr>
<td></td>
<td>pesticides</td>
<td>Dumbaranî (chloropyrrolophenyl)</td>
<td>3.12 µg L⁻¹</td>
<td>adult: no significant effect</td>
<td>-</td>
<td>Ghedira et al., 2011</td>
</tr>
<tr>
<td></td>
<td>metals</td>
<td>Cd</td>
<td>500 µg L⁻¹</td>
<td>adult: no significant effect</td>
<td>-</td>
<td>Ghedira et al., 2011</td>
</tr>
<tr>
<td></td>
<td>drugs</td>
<td>fluoranthene</td>
<td>0.5 - 750 µg L⁻¹</td>
<td>adult: no significant effect</td>
<td>not reported</td>
<td>Ghedira et al., 2011</td>
</tr>
<tr>
<td></td>
<td>PAHs</td>
<td>fluoranthene</td>
<td>2.56 - 100 µg L⁻¹</td>
<td>adult: ≤ 100 (Mus⁺⁺⁺)</td>
<td>+ (Mus⁺⁺⁺)</td>
<td>Rodríguez et al., 2013a</td>
</tr>
<tr>
<td></td>
<td>sediments</td>
<td>-</td>
<td>28d</td>
<td>some contaminated sediments ***</td>
<td>+ (Hp⁺⁺⁺, Zn)</td>
<td>Mosquita et al., 2008b</td>
</tr>
<tr>
<td>IDH</td>
<td>pesticides</td>
<td>Dumbaranî (chloropyrrolophenyl)</td>
<td>3.12 µg L⁻¹</td>
<td>adult: no significant effect</td>
<td>-</td>
<td>Ghedira et al., 2011</td>
</tr>
<tr>
<td></td>
<td>metals</td>
<td>Cd</td>
<td>500 µg L⁻¹</td>
<td>adult: 500 (Hp⁺⁺⁺, gill⁺⁺⁺)</td>
<td>-</td>
<td>Ghedira et al., 2011</td>
</tr>
<tr>
<td></td>
<td>drugs</td>
<td>fluoranthene</td>
<td>0.5 - 750 µg L⁻¹</td>
<td>adult: ≤ 120 (Hp⁺⁺⁺)</td>
<td>not reported</td>
<td>Ghedira et al., 2011</td>
</tr>
<tr>
<td></td>
<td>PAHs</td>
<td>fluoranthene</td>
<td>2.56 - 100 µg L⁻¹</td>
<td>adult: ≤ 40 (Hp⁺⁺⁺)</td>
<td>not reported</td>
<td>Ghedira et al., 2011</td>
</tr>
<tr>
<td>TG</td>
<td>drugs</td>
<td>fluoranthene</td>
<td>0.5 - 750 µg L⁻¹</td>
<td>adult: ≤ 120 (Hp⁺⁺⁺)</td>
<td>not reported</td>
<td>Mosquita et al., 2011</td>
</tr>
<tr>
<td></td>
<td>PAHs</td>
<td>fluoranthene</td>
<td>2.56 - 100 µg L⁻¹</td>
<td>adult: ≤ 40 (Hp⁺⁺⁺)</td>
<td>+ (Hp⁺⁺⁺)</td>
<td>Rodríguez et al., 2013a</td>
</tr>
</tbody>
</table>

Hp: hepatopancreas; Hp*: haemolymph; Mus: muscle; Gor: gonad; ♂: male; ♀: female
**: negative relationship; ***: positive relationship
Significant level: *P≤0.05, **P≤0.01, ***P≤0.005
The organisms' phase II metabolism, which uses biotransformation reactions (also called conjugation reactions), generally serves as a detoxifying step by the addition of molecules naturally present in the body to the toxic molecule, so that it gains a more easily excretable form or achieves the metabolic inactivation of the active compound. The enzymes which catalyse biotransformation reactions are mainly transferases such as GST. The activity of the GST detoxification enzyme was the biomarker used to assess the crabs' phase II metabolism in the reviewed literature (Table 17). The induction of this enzyme was significantly promoted by almost all of the studied pollutants (drugs, PAHs, PCBs, pesticides, and metals), but significant concentration-response relationships were only reported by Aguirre-Martínez et al. (2013b,c) in their studies with environmental concentrations of drugs (caffeine, carbamazepine, ibuprofen, and novobiocin).

<table>
<thead>
<tr>
<th>Table 17 The use of Carcinus sp. to assess glutathione-S-transferase activity in laboratory toxicity studies. Successful outcomes are highlighted.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>drugs</td>
</tr>
<tr>
<td>carboxamipine</td>
</tr>
<tr>
<td>furacilin</td>
</tr>
<tr>
<td>ibuprofen</td>
</tr>
<tr>
<td>novobiocin</td>
</tr>
<tr>
<td>PAHs, PCBs, metals</td>
</tr>
<tr>
<td>cyanobacteria, chlorella</td>
</tr>
<tr>
<td>pesticides, metals</td>
</tr>
<tr>
<td>metal sulfer</td>
</tr>
<tr>
<td>metal sulfer</td>
</tr>
<tr>
<td>metal sulfer</td>
</tr>
<tr>
<td>metal sulfer</td>
</tr>
<tr>
<td>metal sulfer</td>
</tr>
<tr>
<td>metal sulfer + pesticides</td>
</tr>
</tbody>
</table>

*Hp: hypoxanthine, Mau: o-muconate, gH: g-oxalate, Mau: male, g: female
Significant level: *P≤0.001, **P≤0.01, ***P≤0.05

Literature also showed that the responses of Carcinus sp. to pollutants may be assessed through the estimation of specific proteins such as hemocyanin (a respiratory oxygen carrier), stress proteins (e.g., HSP70), metallothioneins (MTs, an intracellular metal regulation protein from which C. maenas's isomers were described by Wong and Rainbow (1986) and Pedersen et al. (1994)), porphyrins...
(PORPHY), as well as vitellin (an egg yolk protein) and vitellogenin (an egg yolk - precursor protein) (Table 18). Generally, specific proteins signal exposure to drugs, endocrine disruptors, PAHs, PCBs, organometals and metals. Nevertheless, concentration-response relationships were only attained between Cu and hemocyanin, as well as between the endocrine disruptor 4-nonylphenol and several metals and vitellogenin like proteins. As shown in Table 18, MTs were the most studied specific proteins, and results corroborate Legras et al. (2000), who found correlations between MTs and soluble essential (Cu and Zn) and nonessential (cadmium, Cd) metal concentrations in C. maenas's Hp and gills. Therefore, results from the present review confirm that MTs are specific proteins of metal regulation on crabs, especially when induced by Cd and Zn. From Table 18 it is possible to conclude that Hm vitellogenin synthesis in Carcinus sp. is a suitable endpoint to assess the effects of environmental concentrations of 4-nonylphenol and metals (Cd, Cu and Zn), with this non-destructive bioassay considered an ethically appropriate biomarker.
Table 18: The use of Carcinus sp. to assess specific-protein levels in laboratory toxicity studies. Successful outcomes are highlighted.

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Exposure chemical</th>
<th>Sensitivity (significantly lower dose or concentration)</th>
<th>Dose–response relationship</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hemocyanin</td>
<td>drugs</td>
<td>diketomethane</td>
<td>10, 100 mg L^{-1}</td>
<td>7d</td>
</tr>
<tr>
<td>metals</td>
<td>Cu (CuCl)</td>
<td>5, 4.2 mg L^{-1}</td>
<td>6d</td>
<td>adult</td>
</tr>
</tbody>
</table>

HSP_{70} MTs (non-specific)

| metals | Cu (CuCl) | 200 µg Kg^{-1} via injection | 14d | adult | (♀) no significant effect | not reported |
| metals | Cu (CuCl) | 750 µg L^{-1} | 7d | adult | (♀) + (Hm) exogenous Cu | not reported |
| metals | Cu (CuCl) | 3 – 100 µg L^{-1} | 14d | adult | (♀) no significant effect | not reported |
| metals | Cd | 3 µg L^{-1} | 7d | adult | (♀) + (Hm) exogenous Cu | not reported |
| metals | Cu (CuCl) | 6.1 – 68.1 µg L^{-1} | 7d | adult | (♀) + (Hm) exogenous Cu | not reported |
| metals | Cu | 15 µg L^{-1} | 21d | adult | (♀) no significant effect | not reported |
| metals | Cu (CuCl) | 200 – 800 µg L^{-1} | 28d | adult | (♀) no significant effect | not reported |
| metals | Zn | 700 µg L^{-1} | 21d | adult | (♀) + (Hm) exogenous Cu | not reported |
| metal mixtures | Cd + Cu | 3 x 15 µg L^{-1} | 21d | adult | (♀) no significant effect | not reported |
| metal mixtures | Cu + Zn | 3 x 700 µg L^{-1} | 21d | adult | (♀) + (Hm) exogenous Cu | not reported |
| metal mixtures | Cu + Zn | 15 x 700 µg L^{-1} | 21d | adult | (♀) + (Hm) exogenous Cu | not reported |
| metal mixtures | Cu + Cu | 3 x 15 x 700 µg L^{-1} | 21d | adult | (♀) + (Hm) exogenous Cu | not reported |
| MTns | metals | Cd | 0.25 – 4 mg Kg^{-1} via injection | 24d | adult | (♀) + (Hm) exogenous Cu | not reported |
| PORPHY | PAHs | BP | 1, 10 µg via injection | 72h | adult | (♀) + (Hm) exogenous Cu | not reported |
| PORPHY | PAHs | BP | 1 – 1,000 µg L^{-1} | 10d | adult | (♀) + (Hm) exogenous Cu | not reported |
| PCBs | endocrine disruptors | MeOH | 1 µg via injection | 72h | adult | (♀) + (Hm) exogenous Cu | not reported |
| vitellin-like | endocrine disruptors | 4-nonylphenol | 1.5, 15 µg L^{-1} | 12w | adult | (♀) + (Hm) exogenous Cu | not reported |
| vitellogenin-like | endocrine disruptors | 4-nonylphenol | 1 – 1,000 µg L^{-1} | 7d | adult | (♀) + (Hm) exogenous Cu | not reported |
| vitellogenin | metals | Cu (CuCl) | 3 µg L^{-1} (CuCl) | 21d | adult | (♀) + (Hm) exogenous Cu | not reported |
| vitellogenin | metals | Cu (CuCl) | 3 µg L^{-1} (CuCl) | 21d | adult | (♀) + (Hm) exogenous Cu | not reported |
| vitellogenin | metals | Cu (CuCl) | 3 µg L^{-1} (CuCl) | 21d | adult | (♀) + (Hm) exogenous Cu | not reported |
| vitellogenin | metals | Cu (CuCl) | 15 µg L^{-1} | 21d | adult | (♀) + (Hm) exogenous Cu | not reported |
| vitellogenin | metals | Cu (CuCl) | 3 µg L^{-1} (CuCl) | 21d | adult | (♀) + (Hm) exogenous Cu | not reported |

Cellular endpoints

The surveyed studies which consider biomarkers at the cellular level are present in Table 19. LMS, usually assessed by the neutral red retention assay (Lowe et al., 1995; Weeks and Svendsen, 1996) or by the neutral red dye uptake method (Babich and Borenfreund, 1991), and the measurement of cellular integrity (i.e., dead or viable) were both considered measures of cellular viability in the present review; and, among others, phagocytic activity (PHAG) was considered a measure of cellular immunocompetence (described on invertebrates by Parry and Pipe (2004)). Also, the ability of an organism to resist oxidative damage (cellular antioxidant capability) is considered a cellular endpoint and is usually attained by measuring lipid peroxidation (LPO) through the thiobarbituric acid method (Wills, 1987), the ferric reducing ability of plasma assay (Benzie and Strain, 1996), or by measuring malondialdehyde (MDA) levels. Cellular antioxidant capability and cellular viability
assessed by measuring LPO and LMS, respectively, were the most used endpoints in the surveyed literature. Both are considered suitable to assess toxicity in *Carcinus* sp., presenting sensitivity to drug compounds. Nevertheless, the successful use of LMS carried out through non-destructive techniques in Hm samples marks this endpoint as a relevant and promising biomarker. Moreover, LPO, measured in Hp, is considered a suitable endpoint to assess Cu contaminated sediment responses of *C. maenas*.

### Table 19: The use of *Carcinus* sp. to assess cellular biomarkers in laboratory toxicity studies. Successful outcomes are highlighted.

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Exposure chemical</th>
<th>Sensitivity (significantly lower dose or concentration)</th>
<th>Dose- or concentration-response relationship</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPO</td>
<td>drugs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>caffeine</td>
<td>0.1 – 50 µg L&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>28d adult &lt;P≥ 0.1 (Hp&lt;sup&gt;*&lt;/sup&gt;, gH&lt;sup&gt;+&lt;/sup&gt;, gH&lt;sup&gt;++&lt;/sup&gt;, Gm&lt;sup&gt;+1&lt;/sup&gt;)</td>
<td>+ (Hp&lt;sup&gt;*&lt;/sup&gt;, gH&lt;sup&gt;+&lt;/sup&gt;, Gm&lt;sup&gt;+1&lt;/sup&gt;)</td>
</tr>
<tr>
<td></td>
<td>carbonazepine</td>
<td>0.1 – 50 µg L&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>28d adult &lt;P≥ 0.1 (Hp&lt;sup&gt;*&lt;/sup&gt;, Gm&lt;sup&gt;+1&lt;/sup&gt;, 50 (Hp&lt;sup&gt;+&lt;/sup&gt;, gH&lt;sup&gt;++&lt;/sup&gt;)</td>
<td>+ (Hp&lt;sup&gt;*&lt;/sup&gt;, Gm&lt;sup&gt;+1&lt;/sup&gt;)</td>
</tr>
<tr>
<td></td>
<td>flunitrazepam</td>
<td>0.5 – 750 µg L&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>7d adult &lt;P≥ 0.1 (Hp&lt;sup&gt;+&lt;/sup&gt;, gH&lt;sup&gt;++&lt;/sup&gt;, Gm&lt;sup&gt;+1&lt;/sup&gt;, Gm&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>+ (Hp&lt;sup&gt;+&lt;/sup&gt;)</td>
</tr>
<tr>
<td></td>
<td>ibuprofen</td>
<td>0.1 – 50 µg L&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>28d adult &lt;P≥ 0.1 (Hp&lt;sup&gt;+&lt;/sup&gt;, gH&lt;sup&gt;++&lt;/sup&gt;, Gm&lt;sup&gt;+1&lt;/sup&gt;)</td>
<td>+ (Hp&lt;sup&gt;+&lt;/sup&gt;)</td>
</tr>
<tr>
<td></td>
<td>novobiocin</td>
<td>0.1 – 50 µg L&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>28d adult &lt;P≥ 0.1 (gH&lt;sup&gt;++&lt;/sup&gt;, Gm&lt;sup&gt;+&lt;/sup&gt;, Gm&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>+ (gH&lt;sup&gt;++&lt;/sup&gt;, Gm&lt;sup&gt;+&lt;/sup&gt;)</td>
</tr>
<tr>
<td>PAHs</td>
<td>fluorethene</td>
<td>2.56 – 100 µg L&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>7d adult &lt;P≥ 2.56 (Hp&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>no significant relationship</td>
</tr>
<tr>
<td>PAHs, PCBs, metals</td>
<td>sediments</td>
<td>-</td>
<td>28d some contaminated sediments**</td>
<td>+ (Hp, Cu)</td>
</tr>
<tr>
<td>metals</td>
<td>pesticides</td>
<td>-</td>
<td>48h adult 3.12 (Hp&lt;sup&gt;+&lt;/sup&gt;, gH&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>metals</td>
<td>-</td>
<td>48h adult 550 (gH&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>-</td>
</tr>
<tr>
<td>PhAG</td>
<td>PAHs</td>
<td>PYR 200 µg L&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>7d Juvenile: &lt;P≥ 1 (Hm&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>Adult: &lt;P≥ 1 (Hm&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>-</td>
</tr>
<tr>
<td>LMS</td>
<td>drugs</td>
<td>caffeine</td>
<td>0.1 – 50 µg L&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>28d adult &lt;P≥ 0.1 (Hp&lt;sup&gt;+&lt;/sup&gt;)</td>
</tr>
<tr>
<td></td>
<td>carbonazepine</td>
<td>0.1 – 50 µg L&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>28d adult &lt;P≥ 1 (Hp&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>neg (Hp&lt;sup&gt;+&lt;/sup&gt;)</td>
</tr>
<tr>
<td></td>
<td>ibuprofen</td>
<td>0.1 – 50 µg L&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>28d adult &lt;P≥ 5 (Hp&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>neg (Hp&lt;sup&gt;+&lt;/sup&gt;)</td>
</tr>
<tr>
<td></td>
<td>novobiocin</td>
<td>0.1 – 50 µg L&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>28d adult &lt;P≥ 1 (Hp&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>neg (Hp&lt;sup&gt;+&lt;/sup&gt;)</td>
</tr>
<tr>
<td>PAHs</td>
<td>fluorethene</td>
<td>2.56 – 100 µg L&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>7d adult &lt;P≥ no significant effect</td>
<td>no significant relationship</td>
</tr>
<tr>
<td></td>
<td>PYR</td>
<td>200 µg L&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>7d Juvenile: &lt;P≥ 1 (Hm&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>Adult: &lt;P≥ 1 (Hm&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>metals</td>
<td>Cu (CuCl&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>6.1 – 68.1 µg L&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>7d adult &lt;P≥ 68.1 (Hp&lt;sup&gt;+&lt;/sup&gt;)</td>
</tr>
</tbody>
</table>

**Significant level: *P≤0.001, **P≤0.01, ***P≤0.05

- neg: negative relationship, +: positive relationship
- *P: 0.001, **P: 0.01, ***P: 0.05

### Physiological endpoints

Several physiological biomarkers were used to determine the responses of *Carcinus* sp. to pollutants, particularly metals (Table 20), namely the evaluation of the changes in heartbeat (measured using CAPMON, a non-invasive infrared light system (Depledge and Andersen, 1990), or the improved AIDA system (Depledge et al., 1996)), the assessment of crabs' osmoregulatory capacity (OC, reviewed by Lignot et al. (2000) in crustaceans), the determination of apparent water
permeability (AWP, reviewed by Rasmussen and Andersen (1996) in crustaceans),
or the measurement of respiration rates. In order not to overload Table 20, the less
frequently used endpoints are mentioned below: studies which considered pollutant
effects on osmoregulation by measuring Hm electrolytes (e.g., Cl\(^{-}\), Na\(^{+}\), K\(^{+}\), Ca\(^{2+}\),
Mg\(^{2+}\)) and studies which considered pollutant effects on the energetic status of
crabs. For instance, Boitel and Truchot (1989) reported no major changes in Hm ion
centralizations of adult male C. maenas in Cu treatments (20-d exposure with initial
single pulse inputs ranging from 0.5 to 2 mg L\(^{-1}\) CuCl\(_{2}\)) when compared to controls.
However, Hansen et al. (1992a) reported that a 7-d exposure of male C. maenas to
10 mg L\(^{-1}\) of copper(II) chloride reduced Hm Na\(^{+}\) levels in crabs collected in January
and in April, whereas Hm K\(^{+}\) concentrations were unaffected in crabs collected in
January and reduced in the ones collected in April. These results coincided with a
reduction in the posterior gill Na\(^{+}\)/K\(^{+}\) ATPase activity (needed for ionic regulation).
Also, Bjerregaard and Vislie (1985) reported a reduction of Cl\(^{-}\), Na\(^{+}\) and K\(^{+}\) in Hm
samples of adult male C. maenas after a 24-h exposure to the lethal concentration
of 10 mg L\(^{-1}\) of Hg, which was also attributed to the inhibition of gill Na\(^{+}\)/K\(^{+}\) ATPase,
as well as to the increase of gill ion permeability. The same study reported that
sublethal centralizations of 0.25 and 0.50 mg L\(^{-1}\) of Hg significantly reduced Hm Na\(^{+}\)
and K\(^{+}\) levels after a 48-h exposure, while 1.0 mg L\(^{-1}\) Hg significantly reduced Hm
Cl\(^{-}\), Na\(^{+}\) and K\(^{+}\). The authors also showed that all lethal and sublethal Hg
concentrations tested induced Hm Ca\(^{2+}\) levels. The energetic status of organisms
may also be used to assess the physiological impact of pollutants, and several
endpoints can be measured, e.g., glucose and lactate levels (overall effect on
glycolysis), total glycogen or lipid content (energy storage), glycolytic enzyme
activities (energy metabolism). For instance, Rodrigues et al. (2013b) studied the
effect of several concentrations of fluoranthene (2.56 to 100 μg L\(^{-1}\)) on adult male C.
maenas by measuring glycogen and lipid content in Hp and muscle samples.
Nevertheless, no significant differences among treatments were observed for any of
the studied parameters. Moreover, Hansen et al. (1992b) concluded that a 7-d exposure to Cu (10 mg L\(^{-1}\) CuCl\(_2\)) significantly reduced male C. maenas's glycolytic enzyme activity (hexokinase, phosphofructokinase and pyruvate kinase). Overall, from the physiological endpoints reported in Table 20 and the ones described above, heart rate monitorisation seems to be one of the most interesting and reliable techniques for researching Cu effects on Carcinus sp., enabling the implementation of non-destructive routine bioassays. All the remaining positive results reported in Table 20 were obtained from concentrations considered not environmental, therefore are not ecologically relevant.
Histomorphological endpoints

Histomorphological biomarkers were also found in this literature survey. Regarding histopathological endpoints, tissue damage can be used, and morphological endpoints can be assessed through biometric alterations (e.g., claw depth, pleopod height, abdomen dimension). For instance, the ultrastructural alteration of adult male *C. maenas*’s gill #8 epithelial cells was studied by Lawson et al. (1995) after a 10-d exposure to Cu (50 μg L⁻¹ Cu(NO₃)₂), showing an extensive ultrastructural change (decrease in the number of plasma membrane infoldings (and associated
mitochondria), extensive vacuolation, a change in ribosomal distribution, and disruption of the microtubular network) in the epithelial layer resulting from the exposure. Moreover, histopathological lesions on adult female *C. maenas* were chosen by Martín-Díaz et al. (2008b) as an endpoint to assess toxicity after a 28-d laboratory exposure to metal contaminated sediment. Histopathological damage was found to be higher in gill tissues, followed by Hp and gonad, and a relationship between metal concentration in sediments and histopathological lesions was observed. Based on the mentioned studies, histopathological damage may be considered an appropriate biomarker to assess metal contamination on *Carcinus* sp.

Behavioural endpoints

Finally, feeding, avoidance, or locomotion abilities are parameters to study behaviour at the individual level; and interspecific interactions such as predator-prey, or intraspecific interactions such as aggregation, territoriality, or social interaction are parameters to study interactive behaviour. Behaviour was also used to assess the responses of *Carcinus* sp. to pollutants. For instance, foraging behaviour, assessed as prey search, prey handling and prey consumption, was the endpoint chosen by Dissanayake et al. (2010) to evaluate the effects of a 28-d exposure to PYR (200 μg L$^{-1}$) of adult male *C. maenas*. Significant differences in prey handling time were observed: PYR-exposed crabs displayed longer prey handling times when compared to control. Moreover, Mesquita et al. (2011) evaluated how adult male *C. maenas*'s locomotion was affected by the antidepressant fluoxetine (0.5 to 750 μg L$^{-1}$) in a 7-d laboratory study. Results showed that locomotion was significantly increased at fluoxetine concentrations equal or above 120 μg L$^{-1}$, with organisms spending more time moving and walking longer distances than control organisms. Of the five locomotor parameters evaluated, two presented a significant concentration-response relationship: the total
time spent moving and the number of segments crossed. Furthermore, Krång and Ekerholm (2006) studied the effect of Cu (100 and 500 μg L$^{-1}$ CuCl$_2$) on specific components of the mating behaviour of male *C. maenas*. Results show that a 5-d Cu exposure clearly altered the crabs’ responses to a pheromone stimulus presented alone, together with a dummy female, or with a real female. Olfactory mediated mating and individual behaviours were significantly disturbed at a 500 μg L$^{-1}$ exposure, and discrimination and courtship behaviours were adversely affected even at the lowest Cu concentration (100 μg L$^{-1}$). The mentioned studies bring together significant work on the behavioural responses of *Carcinus* sp. to PAHs, pharmaceutical drugs and metals, highlighting the potential of this endpoint in toxicity testing using *Carcinus* sp. as a test organism. Also, regarding behavioural endpoints at the individual level, the automated method which has been used to record the behaviour of freshwater invertebrates in their natural sediment, known as Multispecies Freshwater Biomonitor™ (MFB, LimCo International, Germany), was successfully tested in a marine context using *C. maenas* by Stewart et al. (2010). The authors concluded that, when using the MFB, the Green crab appears to be a suitable organism for toxicity testing. Overall, although promising, behavioural biomarkers require further research as environmental concentrations of pollutants are yet to be tested.

Table 21 sums up the selected bioassays, organised by endpoint and pollutant group, which present more potential to be used in *Carcinus* sp. toxicity testing. The pesticide group is the only chemical group which still does not have an associated bioassay. Therefore, further research is needed to clarify the usefulness of *Carcinus* sp. to assess pesticide toxic effects by the biomarker approach. When choosing biomarkers, it should also be taken into account that response evaluation at different levels of biological organisation results in higher quality and more reliable assessments, and that it is through physiological change that an organism regulates
stress responses and alters its behaviour in order to react. Thus, physiological or higher biological endpoints are considered high-level studies, being deemed more ecologically relevant (Connell et al., 1999). Conversely, lower effects are considered early warning signals whose detection can avoid adverse effects at higher hierarchical levels (Van der Oost et al., 2003), which is in compliance with the “precautionary principle” adopted by international policy makers (UNCED, 1992).

### Table 21: Appropriate bioassays for Carcinus sp. toxicity testing by endpoint and pollutant group.

<table>
<thead>
<tr>
<th>Pollutant group</th>
<th>Molecular</th>
<th>Biochemical</th>
<th>Cellular</th>
<th>Physiological</th>
<th>Histopathological</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drugs</td>
<td>alkaline precipitation assay&lt;sup&gt;*&lt;/sup&gt;</td>
<td>EROD&lt;sup&gt;<em>,&lt;/sup&gt; GST&lt;sup&gt;</em>,&lt;/sup&gt; EROD (Hp)</td>
<td>LMS (Hp)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Endocrine disruptors</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PCBs</td>
<td>-</td>
<td>EROD (Hp)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Metals</td>
<td>-</td>
<td>EROD (Hp)</td>
<td>GST&lt;sup&gt;*,&lt;/sup&gt; GR (Hp)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>*</sup> tissue-dependent
<sup>**</sup> see Lawson et al. (1995) and Martín-Díaz et al. (2008b)

**In vitro** studies

**In vitro** studies are well suited for the rapid screening of pollutants, and also for providing a significant amount of information on toxicity mechanisms. Besides, they are inexpensive to carry out and offer ethical advantages. The present review found **in vitro** studies which used *C. maenas* as a test organism to assess the toxic effects of pollutants. For instance, citrate synthase and cytochrome c oxidase enzymes isolated from Hp, gills, and muscle tissue of male *C. maenas* were preincubated **in vitro** with varying Cu concentrations (0.2 to 400 mg L<sup>-1</sup> CuCl<sub>2</sub>) for 30 min. Even though Cu had no significant effect on cytochrome c oxidase activity involved in the final step of oxidative phosphorylation, the study showed a significant reduction of gill and muscle citrate synthase, which controls carbon entry into the tricarboxylic cycle after an exposure to 10 mg L<sup>-1</sup>, while in Hp an exposure to a concentration of 40 mg L<sup>-1</sup> was needed to produce a significant inhibition (Hansen et al., 1992b).

Also, Skaggs and Henry (2002), using subcellular fractions of adult male *C. maenas*'s gill tissue, detected an inhibition of carbonic anhydrase activity (enzyme
that acts in the interconversion of carbon dioxide and bicarbonate) under the influence of metals such as Cd (up to ≈3 mg L\(^{-1}\)), Cu (up to ≈2.3 mg L\(^{-1}\)), silver (Ag, up to ≈500 μg L\(^{-1}\)), and Zn (up to ≈23 mg L\(^{-1}\)). However, these authors found differences in the enzyme’s sensitivity to metal toxicity in the two different species studied (C. maenas and Callinectes sapidus), with the Green crab the most resistant, since it seems to have a metal-resistant isozyme. Moreover, Cu cytotoxicity (≈1 to 5 mg L\(^{-1}\) CuCl\(_2\)) added to an in vitro hepatopancreatic cell preparation was greatly diminished in the presence of Hm proteins, particularly hemocyanin, probably due to a reduced Cu uptake by these cells (Rtal and Truchot, 1996; Rtal et al., 1996). Furthermore, manufactured nanomaterials as titanium dioxide (nano-TiO\(_2\)), nano-Ag and single-walled carbon nanotubes (SWCNT) have already been tested on isolated C. maenas's nerves treated with the nanomaterials. Results showed that there were no effects, at the tested concentrations (≈1 mg L\(^{-1}\)), on the action potential of the nerves of the Green crab (Windeatt and Handy, 2013). In vitro studies are still at an early stage, and the effects of environmental concentrations of pollutants are not yet a priority. Therefore, more research is needed to develop reliable and ecologically relevant in vitro studies derived from crabs. Thus, mitochondrial toxicity assessments and cell-based assays are considered useful topics for future research. Mitochondrial studies integrate physiology in toxicology, so as to determine if, for example, the oxidative or phosphorilative capacities of the organism are altered by the presence of pollutants, which can reduce fitness and, eventually, cause mortality. Mitochondrial studies can also provide an explanation on the mode of action of the tested pollutants. Several studies have already described the isolation of viable mitochondria suspensions of crabs (e.g., Beechey et al., 1963; Chen and Lehninger, 1973; Munday and Thompson, 1962; Poat and Munday, 1971; Siebers et al., 1992). However, to our knowledge, the mentioned approach has not yet been established in ecotoxicological studies with crabs. Similarly, cell-based assays as in vitro models
are becoming a primary topic of interest for aquatic environmental toxicology studies. However, the absence of scientific studies shows that little attention has as yet been drawn to cell cultures of invertebrates. As far as crabs are concerned, the only studies found regarding this subject refer to the successful development of primary, medium- to long-term cultures isolated from haemocytes of the crabs *C. maenas* and *Liocarcinus depurator* by Walton and Smith (1999), and of *C. sapidus* by Li and Shields (2007). In fact, the effect of pollutants on immune system cells could be studied using the abovementioned cell cultures, since haemocytes are the primary effectors of cellular immunity in *C. maenas* (Söderhäll and Smith, 1983). Moreover, a primary Hp cell culture from the edible crab *Scylla serrata* was also described by Sashikumar and Desai (2008). However, no studies were found relating toxicity testing and cell-based assays derived from *Carcinus* sp.

**Field studies**

Assuming that organisms in polluted aquatic ecosystems develop strategies to face the toxicity of chemicals, research on the ecological impact of the toxic levels of chemicals could be carried out in the field. Dose- or concentration-response relationships can also be attained with this kind of studies, which has the advantages of incorporating many of the natural fluctuating environmental conditions and integrating toxicity mixtures naturally present in the field. Table 2 summarises the scientific studies which used *Carcinus* sp. as a sentinel organism in field studies. From these studies, it is possible to acknowledge the successful use of the multi-biomarker approach in order to discriminate different types of pollution or compare reference sites with contaminated ones. Furthermore, this table also shows that higher-level biological biomarkers (physiological, histomorphological, and behavioural) seem to better discriminate environmental quality.
Other types of field studies which expose crabs to pollutants could also be performed, namely transplanting organisms from reference sites or from the laboratory to contaminated sites (also called in situ tests), cross-transplanting organisms and biomonitoring contaminated sites. Moreira et al. (2006), for instance, developed a short-term (48 h) sublethal in situ toxicity assay for estuarine sediment-overlying waters based on postexposure feeding. Then, in order to validate it, the assay was performed in 10 Portuguese reference and contaminated estuaries, where a significant postexposure feeding depression at the contaminated sites was found. Moreover, in order to validate the use of MFO induction as a biomarker to lipophilic contaminants (BP, MeHg and PCBs), Fossi et al. (1998) performed an in situ experiment using C. aestuarii in the Orbetello lagoon (Tuscany, Italy). Results
showed a statistically significant difference between the sites subjected to various human impacts (e.g., industrial effluent, sewage treatment plant, fish farm) when hepatopancreatic BPH activity was measured. Also, sublethal responses in caged *C. maenas* exposed for 28 days to sediments affected by oil spills were evaluated by Morales-Caselles et al. (2008a), who found a link between biomarker measurements (EROD, GPx, GR, GST and histopathological damage) and sediment chemical concentration (metals, PAHs and PCBs). Furthermore, Martín-Díaz et al. (2008a, 2009) concluded that polluted (organic compounds and metals) and non-polluted sediment sites can be easily distinguished by 28-d *in situ* experiments to measure Hp biochemical endpoints (EROD, GPx, GR, GST and MTs) using caged female *C. maenas* placed in four Spanish ports. Additionally, a 28-d *in situ* approach also using caged female *C. maenas* was performed by Buratti et al. (2012), who concluded that LMS was an effective screening tool to detect adverse changes in environmental health status associated with contamination by dredged material (PAHs and metals) in the Algeciras Bay (Spain). Finally, Dissanayake and Bamber (2010) demonstrated that an 8-w *in situ* test using *C. maenas* males of the green morph was an effective way to differentiate PAH-contaminated sites (Algeciras and Gibraltar Bays, Spain) from a reference site (Cadiz Bay, Spain). This study used urine samples and a metabolite fluorescence assay to identify key priority PAH groups (BPs, naphthalenes and PYRs), and LMS and heart rate changes as response criteria.

A 6-d cross-transplanting study was performed by Maria et al. (2009), using male and female *C. maenas* in two sites of Ria Formosa (Portugal), a hypothetical reference site and a metal and PAH contaminated site. Results showed that cross-transplanted crabs (male and female) suffered from pro-oxidant challenges at the contaminated site (assessed as CAT, GR and GPx). Also, GST was reduced in both gender crabs transplanted from reference to contaminated places. Even though MT
induction occurred in crabs transplanted to the contaminated site, differences were also observed in gender and organ specificities (Hp and gills). Moreover, in what concerns damage, LPO and genotoxicity only manifested themselves in the gills of male crabs transplanted to the contaminated site.

The health of aquatic ecosystems can also be estimated through the performance of biomonitoring programmes using Carcinus sp. as a sentinel organism. However, Orbea et al. (2002) pointed out that in order to achieve toxicity patterns over time, studies should take into account seasonal differences. These authors concluded that seasonal factors can affect biomarker responses to a greater extent than pollution variations. Moreover, Dissanayake et al. (2011) found seasonal variations in the C. maenas's physiology of the organisms collected in a reference site. No biomonitoring studies assessing environmental quality over the years were found for Carcinus sp.

**Indoor and outdoor mesocosm experimental data**

The use of laboratory experimental designs involving sublethal concentrations and also species of different trophic levels exhibiting various feeding strategies (e.g., filter feeding, grazing, omnivore, predation) enhances the relevance of ecotoxicological studies. These multi-species toxicity assessments take into account different routes of exposure, ecological roles and metabolic capabilities of organisms, thereby providing a more realistic and suitable “diagnosis of stress”, as was highlighted, among others, by Brown et al. (2004), Galloway et al. (2004b) and by the ECOMAN approach (Galloway et al., 2004a, 2006). Furthermore, pollutants may also alter the structure of a community or ecosystem. This becomes noticeable when parameter shifts such as the number of species are observed. Therefore, in order to attain ecological relevance, experimental control and replication, the BEEP project (Biological Effects of Environmental Pollution in Marine Ecosystems)
developed a 3-w indoor mesocosm experimental study where different species (*C. maenas, Gadus morhua* and *Scophthalmus maximus*) were exposed to nonylphenol, North Sea crude oil and a combination of crude oil and alkylated phenols (*Sundt et al., 2006*). The aim of the project was to validate and intercalibrate a battery of biomarkers in selected indicator species. The outcomes of the BEEP project highlighted the use of MN (gills), AChE (gills), MTs (Hp) and LMS (Hp) as relevant biomarkers to monitor the biological effects of pollutants under marine environmental monitoring and assessment programmes in Europe and the US (*Lehtonen et al., 2006*). Moreover, *C. maenas* and *Mytilus edulis* were used in a mesocosm experiment aiming to compare its results with those from a natural pollution gradient (field study). However, the endpoints studied, lipid content and lipid/protein ratio, showed differences in both studies which were attributed to differences in temperature exposure (*Capuzzo and Leavitt, 1988*). Furthermore, in an outdoor mesocosm experiment involving diesel oil and Cu dosing, no significant effects were observed in *C. maenas* hepatopancreatic GST, nor in the digestive gland of *M. edulis*. However, *Littorina littorea* showed significantly higher GST activity at the highest concentration treatment (*Lee, 1988*).

**The use of indices in laboratory and field toxicity studies**

Biomarker-based indices have been proposed as useful tools to achieve a comprehensive evaluation of the impact of pollutants, reflecting their effects on different endpoints. Therefore, two stress indices aiming to assess aquatic environmental impairment using *Carcinus* sp. as a test organism were found: the Integrated Biomarker Response (IBR) (*Beliaeff and Burgeot, 2002*) and the Biomarker Response Index (BRI) (*Hagger et al., 2009*). The latter index was advanced as a tool to monitor the condition of England’s Special Marine Areas of Conservation under the Habitats Directive (*Directive 92/43/EEC*). In laboratory
studies, this approach could allow for the comparison and ranking of the effects induced by exposure to a number of pollutants. However, in this literature survey, no laboratory studies were found which used the mentioned approach in *Carcinus* sp. Nevertheless, this approach was recently and successfully used by Parolini et al. (2013) to rank the toxicity of five pharmaceutical drugs and personal care products using the bivalve *Dreissena polymorpha* as a test organism in a short-term laboratory study. The authors concluded that the application of the BRI successfully decreased biomarker variability and enabled the toxicity ranking of the tested chemicals. On the other hand, the application of this index in a field context allowed for a good discrimination between different polluted sites in the Bizerta lagoon (Tunisia), and was also in line with the chemical analysis carried out on sediment (Ben-Khedher et al., 2013a). Moreover, Jebali et al. (2011) considered the IBR index as a useful tool in the monitoring of the Tunisian coast, and Pereira et al. (2011) concluded that the same index revealed “stressful” conditions in crabs at the studied contaminated sites (Óbidos lagoon, Portugal), even though results were seasonal- and gender-dependent. It is possible to conclude that both BRI and IBR may successfully be applied in a field context in order to discriminate environmental impairment. However, natural variation in biomarkers over time should be taken into account in order to perform a suitable discrimination of sites, as was highlighted by Orbea et al. (2002) and Dissanayake et al. (2011).

Several other indices may be calculated in order to assess different and complementary information concerning the impairment caused by pollutants. For instance, a measure to integrate the physiological status of organisms initially proposed for *M. edulis* by Widdows and Johnson (1988), the Scope For Growth (SFG, J mg dw$^{-1}$ h$^{-1}$) index, is an energetic status indicator determined from the energy absorbed from ingested food items minus the energy lost through respiration and excretion. The SFG index was only applied by Dissanayake et al. (2008a) to
juvenile and adult male *C. maenas* exposed to PYR, and results showed that the SFG index only decreased significantly in the adults as an effect of PYR exposure. Therefore, the confirmation of the limitations of the SFG index in what concerns its use in juveniles is necessary, since the organism growth process seems to superimpose their response to pollutants.

From a morphological point of view, increased organ wet weight/dry weight ratio (ww/dw) in decapod crustaceans has been reported to reflect the general health status of an organism. However, the application of *C. maenas's* hepatopancreatic ww/dw did not reveal the metal gradient found in the sediments by Pedersen and Lundebye (1996), and Lundebye and Depledge (1998a,b) found no significant differences between crabs exposed to Cu (200 to 800 μg L⁻¹ CuSO₄) and control crabs. On the other hand, a 7-d exposure to Cu (10 mg L⁻¹ CuCl₂) of male *C. maenas* collected in May significantly increased Hp and muscle ww/dw ratios, while it revealed no effects on gills (#7, #8 and #9) ww/dw ratio. However, results were seasonal-dependent, since Cu exposure had no effect on ww/dw ratios (Hp, gills and muscle) in crabs collected in September or October (Hansen et al., 1992a). Therefore, further research is necessary to clarify ww/dw index sensitivity so as to detect the effects of pollutants on *Carcinus* sp. Moreover, morphological indices which identify possible organ diseases can also be calculated: the gonadosomatic index (GSI), hepatosomatic index (HSI) and gill somatic index (Gill-SI) are all determined by the ratio between organ weight (g) and body weight (g). Thus, Lye et al. (2008) provided evidence that GSI and HSI parameters in male *C. maenas* were significantly modified by exposure to the xenoestrogen 4-nonylphenol. Also, Elumalai et al. (2005) observed a concentration dependent reduction of both GSI and HSI in adult female *C. maenas* exposed to metals (Cr, Cu, and their mixtures). Nevertheless, Fossi et al. (1996) estimated the HSI and Gill-SI of *C. aestuarii* after exposure to BP, MeHg and PCBs, reporting that, at that time, the use of these
indices required further research. To conclude, indices which detect organ diseases seem to be sensitive to endocrine disruptors and metals, and further studies shall be required to test other chemical groups.

At the individual level, and according to Pereira et al. (2006), organisms may exhibit reduced values of the condition index (CI), estimated by the ratio between organisms' wet weight (g) and cephalothorax width (cm), as a result of environmental impairment. Therefore, Mesquita et al. (2011) calculated this index in order to assess whether adult male *C. maenas* exposed for 7 days to different concentrations (0.5 to 750 μg L⁻¹) of fluoxetine (anti-depressant) were in similar health conditions. However, no significant differences were observed at the end of the test. Moreover, Pereira et al. (2008) found no CI differences in what concerns female *C. maenas* between two sampling sites in the Óbidos lagoon (Portugal), one considered as a reference site and the other a contaminated (metals and nutrients) site. As for this index, and in agreement with the results obtained in their study using adult male *C. aestuarii* as a test organism, Ricciardi et al. (2010) suggested that further studies are necessary to clarify the relationship between CI and environmental stress due to pollutants.

**Towards a realistic assessment of *C. maenas*'s toxic effects**

Individual variations of the test organisms such as genetics, gender, size, morphotype, stage of the moulting cycle, nutritional status and health condition may affect the organisms' absorption, distribution, metabolism and excretion competences, thus becoming confounding variables in ecotoxicity assessment. Moreover, previous or concurrent exposure to pollutants may induce differential sensitivity to further contamination. Furthermore, experimental factors, such as temperature, salinity or pH can affect the organisms (e.g., metabolic rates, behaviour), the chemicals (e.g., activity, degradation), and the possible interaction
between both. Therefore, in order to produce reliable cause-effect relationships and to reduce uncertainty in the results, all these variables should be taken into account in ecotoxicity assessment. The purpose of the following sections is to identify the tendencies of these potentially confounding variables so as to observe their influence on *C. maenas*'s responses to pollutants, thus drawing attention to the importance of harmonisation for data collection.

Gender, size and morphotype

*Carcinus* sp. is sexually dimorphic, e.g., body and chela dimensions, abdomen size and shape (abdomen of males is triangular, whereas that of females is broader and rounder), pleopod structure (pleopods of males are modified for copulation and those of females are used to hold eggs), which represents an advantage in the assessment of eventual gender-specific responses. For instance, even though results showed significant effects on the crabs' Hm osmolality and OC after a 7-d exposure to the drug diclofenac (10 and 100 ng L⁻¹), *Eades and Waring* (2010) found no significant effects in terms of gender in their results. On the other hand, in a field survey, *Brian* (2005) found significant differences in the degree of heterochely (morphological endpoint) expressed by male and female *C. maenas* in response to endocrine disruptors. Also, from a cross-transplanted study, and with the intent to evaluate environmental contamination by hydrocarbons and metals in the field, *Maria et al.* (2009) demonstrated that GR, MTs and genotoxicity responses were significantly different in males and females. However, these differences were attributed to the female reproductive state, since the majority of females captured were ovigerous. Hence, the same study concluded that female crabs were more able to cope with pro-oxidant pollutants, since LPO levels in female crabs decreased in the gills and Hp, whereas in males it increased in the gills after exposure to the mentioned chemicals. In what concerns pesticides, *Gowland et al.*
(2002) found no differences between genders after exposing adult *C. maenas* to several concentrations of the pyrethroid cypermethrin. Conversely, Pereira et al. (2009, 2011) found gender specificities in the antioxidant system of *C. maenas* which were attributed to metal contamination and eutrophication, with females being more vulnerable. Furthermore, significant sex-related differences upon exposure to calcium hydroxide were found in *C. maenas* by Cripps et al. (2013), with females also more susceptible. Thus, it is possible to conclude that gender may produce distinct ecotoxicological results and should be taken into account in the planning of experimental designs and in the comparison of results.

Ecotoxicological comparisons between *Carcinus* sp. of different sizes were not well documented, as only two studies were found: Dissanayake et al. (2008a) revealed physiological differences between juvenile and adult male *C. maenas* (green morph), with juveniles significantly more susceptible to the effects of PYR exposure since they showed lower immunocompetence, lower metabolic energy and increased SFG index, when compared to adults. Also, significant size related differences upon exposure to calcium hydroxide were found by Cripps et al. (2013), with mature females the most susceptible. Accordingly, ecotoxicological studies should also take into account the size of test organisms.

Despite its name, the Green crab may have two observed colour morphotypes, green and red (light-orange to deep-red), with the distinction based on abdomen colouration. A synthesis of studies reporting the main differences between the two morphs was carried out by Reid et al. (1997). Briefly, the green morph is better suited to cope with environmental stress such as salinity fluctuations and low oxygen levels, while the red colouration has been associated with prolonged intermoult periods, conferring an advantage to males when intense competition for females occurs, since they have thicker carapaces and more robust chelas when compared to similar-size green morph. Moreover, the green morph can usually be
found in intertidal areas whereas the red morph prefers subtidal habitats. In order to study whether both warming and acidification would affect the long-term (5 months) individual performance of adult male *C. maenas*, and whether the effects were morph-dependent, Landes and Zimmer (2012) carried out an experiment whose endpoint was interspecific interactions. Although effects of acidification were identified, these authors did not detect any significant differences between the two morphs. On the other hand, field experiments in a weakly tidal fjord (Isefjord, Denmark) have shown that tidal rhythms are more frequently observed in green morphs than in red morphs (Styrishave et al., 1999). Additionally, ecotoxicological comparisons between green and red morphs were found in this literature survey, since these two morphotypes respond differently to the same pollutant. For instance, Rewitz et al. (2003) studied two CYP isoform enzymes, CYP330A1 (closely related to members of the CYP2 family) and CYP4C39 (identical to crayfish CYP4C15), on adult male *C. maenas* after injection with a barbiturate drug (phenobarbital) and with the steroid hormone ecdysone. The authors concluded that the CYP330A1 gene expression was only induced in the Hp of the green morph by both phenobarbital and ecdysone, which means that, when compared to the red morph, the green morph presents a higher possibility of conversion by phase I metabolism. However, the mentioned compounds affected neither the green nor the red morphs' CYP4C39 gene expression. Moreover, Dam et al. (2006), who studied PYR toxicity on adult male *C. maenas*, showed that red crabs exposed to PYR had a significantly higher mortality rate (100% within 51 d) than any other studied group (green morph exposed to PYR, solvent controls for green and red morphs, and controls for green and red morphs). This study also showed an increasing abundance of CYP transcripts in green crabs, when compared to the red ones, which indicates a higher rate of PYR conversion into the Green crabs' phase I metabolite 1-hydroxypyrene. As explained before, CYP enzymes play a pivotal role in the initial phase I metabolism of lipophilic pollutants such as PYR, and the green
Morph seems to have a higher abundance of CYP enzymes. Furthermore, in the same research study, \textit{in vitro} pyrene hydroxylase assays revealed significantly higher NADPH-dependent pyrene hydroxylase activity in the hepatopancreatic microsomes of green crabs, when compared to the red ones. As for metals, the influence of Cd accumulation in the composition of fatty acid of both morphs of \textit{C. maenas} was assessed by Styrishave et al. (2000), who found that the green morph is more tolerant to Cd exposure than the red morph. Also, Krång and Ekerholm (2006) studied the influence of Cu in the ability of male crabs to detect and search for females (mating behaviours). Parameters such as olfactometer search and discrimination and courtship performance were evaluated in this study. However, with the exception of decreased stroking for red crabs, no such selective colour morph sensitivity to Cu was found. The same result was attained by Landes and Zimmer (2012) in their long-term (5 months) exposure of male \textit{C. maenas} to acidified seawater (pH 7.7). Data gathered from the mentioned studies shows morphotype-dependence in some of the results, highlighting the importance of considering this item in the experimental design and when comparing results. It should be noted that most of the surveyed studies did not specify the \textit{Carcinus} sp. morphotype used.

Moult cycle

Growth in \textit{Carcinus} sp. is associated with the moult cycle because of the limitations imposed by the calcified exoskeleton. The major secretory products of \textit{C. maenas} moulting glands are ecdysteroids ecdysone and its 25-deoxy form (25-deoxyecdysone). Released ecdysteroids are further hydroxylated by peripheral tissues at the 20-position, with 20 hydroxyecdysone and ponasterone A the 20-hydroxylated metabolites of ecdysone and 25-deoxyecdysone, respectively (Lachaise et al., 1986). Therefore, 20-hydroxyecdysone and ponasterone A were
the major ecdysteroids detected in crab Hm (Lachaise and Lafont, 1984). In general, the level of ecdysteroids in crustaceans' Hm is low throughout intermoult (stages C1-C4), rises during premoult (stages D0-D4), typically peaks in D2-3, and then falls prior to moultng, presenting low levels during ecdysis (stage E, moultng process) and postmoult (stages A-B) (Skinner, 1985). Also in male crab Hp, ecdysone, 20-hydroxyecdysone and ponasterone A varied over the moult cycle, with high levels in premoult and low levels in postmoult and intermoult (Styrishave et al., 2004). The same pattern was observed in female hepatopancreatic ponasterone A, but ecdysone and 20-hydroxyecdysone remained high from early intermoult (C1) until late premoult (D3) and only decreased during postmoult (Styrishave et al., 2008). Concerning the gonads (C. maenas testis), high levels of ecdysone and 20-hydroxyecdysone were present, with the exception of postmoult (stage A), and ponasterone A was never observed by Styrishave et al. (2004). As abovementioned, CYP enzymes play a pivotal role in phase I metabolism of pollutants, but some of the CYP enzymes are also directly responsible for the biosynthesis of the previously referred steroid hormones. Accordingly, Dam et al. (2008) studied the expression of six CYP genes in the Hp of C. maenas and concluded that the expression of all the studied genes was predominant during postmoult and intermoult stages but, in general, these genes had low expression in the premoult stage. Also, Rewitz et al. (2003) and Styrishave et al. (2004) showed that the levels of ecdysteroids and specific CYP mRNAs in C. maenas varied throughout the moult cycle. Therefore, both pollutants which can be metabolised by phase I reactions and may affect endocrine functions, and pollutants which are able to disrupt the endocrine system itself (endocrine disruptors) may have consequences on Carcinus sp. ecdysis. The influence of calcium (Ca), Cu and Zn was also studied during the moult cycle of adult male C. maenas by Scott-Fordsmand and Depledge (1997). The authors found that Ca was lost from Hm during premoult and was apparently stored in the Hp, and at ecdysis the Hm Ca content doubled. During early postmoult, the Ca
stored in the Hp was used in combination with the Ca absorbed from the surrounding seawater for a rapid calcification of the new exoskeleton. Concerning Cu and Zn concentrations, they both declined in the “rest tissue” compartment during premoult, and, at ecdisis, Hm Cu and Zn contents increased from premoult to the newly moult stage. During postmoult, Cu and Zn were lost from Hm and mainly excreted but for a minor fraction (5-10%) which was stored in the Hp.

Depending on the moult stage, organisms may have different sensitivities to pollutants. Hence, having this in mind, and in order to report the effects of pollutants over the moult cycle in Carcinus sp., some studies are presented below. For instance, after studying how adult male C. maenas handles Ca, Cu and Zn over the moult cycle, Scott-Fordsmand and Depledge (1997) concluded that an exposure to raised ambient dissolved Cu (100 μg L\(^{-1}\) CuCl\(_2\)) during ecdisis and postmoult may have an effect on postmoult Ca content, which is essential for exoskeleton formation. Results showed that in Cu-exposed crabs, hepatopancreatic Ca and Zn were reduced, while Cu increased in the Hp and in other tissues. Even though Ca storage in the Hp constitutes a small fraction of the total intermoult Ca content, it appears to be important for the immediate recalcification of the exoskeleton following ecdisis. It was then concluded that at ecdisis and during postmoult, crustaceans exhibit increased sensitivity to Cu. Moreover, Mesquita et al. (2011) studied the effect of fluoxetine (anti-depressant) on the adult male C. maenas moult cycle by assessing the levels of N-acetyl-b-glucosaminidase in the epidermis, an enzyme which plays a functional role on the moult process. Nevertheless, the authors concluded that this enzyme varied over the moult cycle but no alterations were observed after exposure to fluoxetine. On the other hand, Lye et al. (2008), who investigated the impact of 4-nonylphenol, a precursor to commercial detergents, on the regulation and functioning of the endocrine system of male C. maenas, concluded that an effective concentration of 15.7 μg L\(^{-1}\) of 4-nonylphenol
significantly decreases the level of Hm ecdysone, which could lead to a permanent state of intermoult. Moreover, fluctuations of phenobarbital (barbiturate drug) and PAH effects over the moult cycle were also reported by Dam et al. (2008). Results showed that Hp CYP enzyme activities might be reduced during premoult and ecdysis, impairing the crabs' ability to metabolise PAHs in these life stages. Consistent with the latter, Dam et al. (2006) also observed an increased mortality of premoult crabs injected with PYR when compared to intermoult crabs. Therefore, the moult stage of crabs is a parameter to be taken into account, especially with regard to endocrine disruptors toxicity testing.

Nutritional status

In addition to a broad physiological tolerance, *Carcinus* sp. are voracious omnivores and opportunistic in their feeding habits (Donahue et al., 2009). Moreover, their tolerance to starvation also conveys them adaptability and hardiness characteristics. For instance, at 10°C, adults can survive for three months without food, with only a 50% mortality rate (Wallace, 1973). Nevertheless, nutritional conditions should not influence the response criteria determined in ecotoxicology scientific research. Therefore, some studies researched the feeding rates of *Carcinus* sp., as well as the influence of starvation on the crab's susceptibility to pollutants. For example, the influence of gender on the feeding rate of juvenile *C. maenas* during a 30-min. feeding period with dried pieces of the polychaeta *Hediste diversicolor* was studied by Moreira et al. (2006), but no significant differences were found between green colour-form male and female feeding rates. The same study also found similar feeding rates after crabs had been subjected to a starvation period of 48, 96 and 192 hours. Moreover, with the intent to study the influence of starvation in adult male *C. aestuarii*, Matozzo et al. (2011a) demonstrated that a 7-d starvation period influenced the immune parameters of the crab (assessed as total haemocyte count,
haemocyte diameter and volume, haemocyte proliferation, cell-free Hm, glucose and total protein (TP) levels, and phenoloxidase (PO) activity), but did not induce oxidative stress (assessed as CAT and SOD activities). Results also indicated that *C. aestuarii* can modulate its cellular and biochemical parameters in order to cope with starvation. Furthermore, Wallace (1973) studied the metabolic level of starved *C. maenas* measured by the amount of consumed oxygen, concluding that this endpoint dropped by 40% during the first week of starvation, then remaining steady for the next two weeks, before dropping again another 20%, where it remained for a further 9-w period.

In what concerns the influence of starvation on crabs' susceptibility to pollutants, Dissanayake et al. (2008b) showed that adult male *C. maenas* (green morph) were relatively robust when facing nutritional changes (starved, fed on alternate days and fully fed), presenting no significant differences in terms of physiological condition between the control group and the 7-d PYR-exposed crabs. However, after 14 days, starved crabs showed significant impacts on their condition, presenting a significantly lower antioxidant status, when compared to crabs under both types of feeding regimes. The same study also concluded that the urine of starved crabs had significantly higher levels of proteins than that of fed or diet-restricted individuals, indicating that starved individuals displayed proteinuria. A subsequent study by the same authors, with the same experimental conditions, showed that a reduced physiological condition in starved PYR-exposed crabs resulted in an unexpected increase in their competitive ability (measured as agonistic interactions) when compared to non-starved individuals (Dissanayake et al., 2009). Furthermore, the effects of a 40-d cadmium chloride exposure and dietary status (starved, fed) on Cd accumulation and fatty acid composition were analysed by Styrishave et al. (2000) in the two morphotypes of *C. maenas*. The authors concluded that the green morph is more tolerant to starvation and Cd exposure than the red morph. In conclusion, in
laboratory sublethal assays with longer exposure regimes, nutritional status can influence the crabs' susceptibility to tested chemicals. Therefore, to produce reliable results, ecotoxicological studies, especially long-term ones, should take the crabs' nutritional condition into account.

Historical or concurrent contamination

Historical and concurrent contamination are a challenge to toxicity testing since organisms may acquire resistance to pollutants and responses may be due not to the pollutant itself but to other concurrent factors (e.g., temperature). Accordingly, *C. maenas'*s resistance to the organophosphate pesticide fenitrothion was analysed by Rodrigues et al. (2013a) in a laboratory study. These authors found that adult male crabs previously collected from a moderately contaminated estuary (Lima estuary, Portugal) were less sensitive to fenitrothion, showing lower AChE inhibition, than those collected from a low impacted estuary (Minho estuary, Portugal). Other biomarker changes detected in the crabs collected from the moderately contaminated estuary were: increased anaerobic metabolism (muscle lactate dehydrogenase) and enhanced GST, GR, GPx, CAT and TG. No other relevant studies focusing on historical or concurrent contamination affecting the responses of *Carcinus* sp. to pollutants were found.

Experimental temperature, salinity and pH

The effects of temperature on crabs' cellular and biochemical parameters were evaluated by Matozzo et al. (2011b) in a laboratory experiment. These authors found that the adult male *C. aestuarii* modulated its cellular and biochemical parameters (mainly haemocyte proliferation, cell-free Hm protein concentrations and cell-free Hm phenoloxidase activity) in order to cope with thermal stress (4°C and 30°C). In what concerns the toxic levels of chemicals in general, the higher the
temperature, the greater the effects of toxicity in a biological system will be. Actually, Camus et al. (2004) concluded in their laboratory work that heart rate in adult male \( C. \) \textit{maenas} of the green morph was more vulnerable to Cu contamination at temperature extremes (5°C and 25°C) than at the standard temperature of 15°C, and an enhanced Cu toxicity was observed at 25°C by the measurement of an erratic heart rate. Moreover, in a field study carried out by Pereira et al. (2011) in a moderately contaminated coastal system (Óbidos lagoon, Portugal), the authors observed that seasonal differences of \( C. \) \textit{maenas}'s biochemical responses (CAT, EROD, GPx, GST, LPO and TG) superimposed spatial variations. Winter enzymatic increases were in agreement with a higher availability of metals in water and an enhancement of Hp accumulated levels, while increases in summer were mainly driven by non-contamination related factors (e.g., water temperature).

The work of Rainbow and Black (2001) showed that under certain conditions, adult crabs are able to alter its apparent water permeability (AWP) in response to decreases in salinity, with this capacity a physiological control mechanism to osmotic stress. However, it is known that water salinity fluctuations may alter vital processes on \( \text{Carcinus} \) sp. (Henry et al., 2003; Martín-Díaz et al., 2004b), as well as behaviour (McGaw et al., 1999). Accordingly, water salinity should also interfere with the crabs' responses to pollutants. It is also known that \( \text{Carcinus} \) sp. is perfectly able to regulate low concentrations of essential metals (e.g., Cu, iron (Fe), magnesium (Mg), Zn) by physiological responses, even at low salinities (e.g., Rainbow and Black, 2002). Nevertheless, metal toxicity may occur under certain conditions, with higher effects usually associated with low salinities. For instance, the effects of Cd (0.5 to 8.0 mg L\(^{-1}\) CdCl\(_2\)) and Cu (2.5 to 40 mg L\(^{-1}\) CuCl\(_2\)) on \( C. \) \textit{maenas} and \( \text{Cancer} \) \textit{irroratus} under five different salinities were studied by Thurberg et al. (1973) during a 48-h exposure experiment. Parameters such as Hm osmolality, OC and respiration rates of gill-tissue were measured. The authors
concluded that Cd causes an elevation of Hm osmolality in *C. maenas*, and a depression of gill-tissue oxygen consumption in both species, and that higher oxygen consumption values were observed at low salinities. Concerning Cu effects, a disruption of the osmoregulatory system in both species was reported, being more pronounced at lower salinities, and no Cu effect was observed in gill-tissue oxygen consumption. Also, Weeks et al. (1993) found that a decrease in salinity from 20 to 10 had no effect on the acid-base status of adult male *C. maenas*, and an increase in salinity from 20 to 30 caused a minor metabolic acidosis in Hm. However, the degree of depression of Hm pH after a 7-d exposure to Cu (750 μg L\(^{-1}\)) was salinity-dependent, with the most severe acidosis occurring in Cu-exposed crabs at 10. These authors also reported that lowered salinity combined with a Cu stress enhanced the rate of Cu removal from the Hm to the Hp. Hansen et al. (1992a) also recorded synergistic effects between low salinity and a 7-d maximum exposure to Cu (10 mg L\(^{-1}\) CuCl\(_2\)), since a 50-60% reduction in male *C. maenas*'s posterior gill Na\(^+\)/K\(^+\) ATPase activity at 10 salinity was reported. Furthermore, Legras et al. (2000) found that the MT level was related to metal concentrations (Cd, Cu and Zn) in both Hp and gills of *C. maenas*; and that metal concentrations were inversely related to salinity in the Gironde estuary (France). Nevertheless, the typical response of higher metal toxicity as salinity decreases was not observed by Lawson et al. (1995), who studied the effect of Cu (50 μg L\(^{-1}\) Cu(NO\(_3\))\(_2\)) under two different salinities (10 and 35) on the ultrastructure of the gill epithelium of adult male *C. maenas*. Results showed that Cu caused extensive alterations, including a decrease in the number of plasma membrane infoldings (and associated mitochondria), an extensive vacuolation, a change in the ribosomal distribution and a disruption of the microtubular network. However, the extent of the changes was greater at 35 than at salinity of 10. Finally, a more comprehensive field study was developed by Rodrigues et al. (2012) with the intent to compare the effect of salinity fluctuations (4 to 45) on adult male *C. maenas* collected in a reference and a contaminated (PAHs,
metals and nutrients) estuary, the Minho and Lima estuaries (Portugal), respectively. Results showed that salinity change superimposed higher stress on crabs collected in the contaminated estuary when compared to the ones collected in the reference estuary. Endpoints such as neurotransmission (ChE), energy metabolism (IDH) and lactate dehydrogenase (LDH), biotransformation and antioxidant defences (GST, GR, GPx and TG), as well as oxidative damage (LPO) were significantly altered on the crabs collected in the contaminated estuary, while only altered neurotransmission and antioxidant (GR) defences produced by salinity fluctuations were measured in the crabs collected at the reference estuary. Therefore, it is possible to conclude that metal uptake and disruption vary with changes in salinity, with usually lower salinities associated with increased toxic effects. These results are in line with the fact that chloride complexation decreases in low salinity values (Bruland, 1983), resulting in a possible increase in free metal ion concentration. Moreover, it is known that, at lower salinities, crabs require greater capacities of the osmoregulatory system, and that *C. maenas* is considered a modest osmoregulator when compared to other crabs, e.g., *C. sapidus* (Kotlyar et al., 2000). Thus, since processes of osmoregulation involve metabolic work, increased energetic demands at lower salinity may also decrease the tolerance of *Carcinus* sp. to metals. No other pollutant groups were found in literature in what concerns the influence of salinity on crabs' responses to pollutants.

The increase in atmospheric carbon dioxide driven by anthropogenic activities is known to result in ocean hypercapnia, which consequently decreases seawater pH and shifts carbonate speciations. Therefore, deleterious impacts of hypercapnia and acidification on *C. maenas* have been studied by several authors. For instance, Fehsenfeld et al. (2011) considered that *C. maenas* exhibits only a weak overall response to hypercapnia (assessed by gill gene expression determinations), and Appelhans et al. (2012) concluded in their study on feeding behaviour that *C*. 
*maenas* only feed less under strong acidification ($p\text{CO}_2$ 354.6 Pa). They also concluded that *C. maenas* can actively compensate extracellular pH by means of bicarbonate accumulation, with anterior gills more efficient in elevating pH than posterior gills, among which the anterior gill #4 has the highest proton excretion rate (*Fehsenfeld and Weihrauch, 2013*). Significantly elevated Hm potassium and ammonia concentrations under hypercapnia ($p\text{CO}_2$ 324.3 Pa) and an increased ammonia excretion rate were also observed by the same authors. Furthermore, *Hammer et al. (2012)* concluded that a 4-w exposure to elevated levels of carbon dioxide ($p\text{CO}_2$ 770 Pa) might impair intracellular iso-osmotic regulation, since a general decrease in the majority of intracellular osmolytes was found after the use of a metabolomic-based tool (nuclear magnetic resonance-mass spectrometry, NMR-MS). On the other hand, acidification negatively affected the closer-muscle length of the crusher chela and, correspondingly, the claw-strength increment in adult male *C. maenas* (*Landes and Zimmer, 2012*). However, the same study showed no evidence that predator-prey interactions will change in future acidification scenarios, since acidification affected both predator claws (*C. maenas*) and prey shells (*L. littorea*). No studies were found in the present review relating hypercapnia and acidification with pollutants in what concerns the responses of *Carcinus* sp. Nevertheless, research in this area is a current and relevant trend, and there are still many unanswered questions, e.g., the possible decalcification of crabs’ exoskeleton due to decreased pH. However, the physiological effects of the use of chemical sequestration on *C. maenas* as an alleviation strategy to reduce the impacts of ocean acidification have already been studied by *Cripps et al. (2013)*. Results showed that a 6-h exposure to calcium hydroxide significantly affected tested organisms’ acid-base balance, causing slight respiratory alkalosis and hyperkalemia. Therefore, enhanced alkalinity to counteract ocean acidification needs to be further studied in order to clarify the possible effects of this remediation strategy on living beings.
Concluding remarks

Studies in aquatic ecotoxicology aim at filling out the gap between routine laboratory chemical analyses and the complexity of the natural environment. Currently, the level of pollutants in aquatic ecosystems is increasing, thus becoming one of the most relevant stressors that aquatic organisms have to face on a daily basis. Studies involving physical, chemical and biological factors are considered highly relevant, as they contribute to better and more accurate outcomes. Moreover, the selection of adequate animal experimental models and experimental designs is of major importance to obtain scientifically valid results both in the laboratory and in the field. Thus, this literature review concluded that *C. maenas* is not a sensitive organism in terms of mortality assessment. Nevertheless, toxicity tests based on mortality are currently considered as having restricted value for, in the environment, most pollutant exposures are at sublethal levels. On the other hand, the use of biomarkers to measure and analyse the effects of pollutants on crabs in an integrative perspective is emphasised by the results of the present review. A large number of toxicological endpoints, from molecular to behavioural levels, were studied and several have already been validated. Therefore, the gathered data enabled the selection of adequate and reliable bioassays to be used in *Carcinus* sp. toxicity testing, which are presented in Table 21. Moreover, the present review concluded that the biomarker approach was also successfully used to discriminate natural sampling sites according to types of environmental pollution, reflecting the different conditions of anthropogenic impacts. Also, the ECOMAN project, based on the biomarker approach and developed to assess the general health of estuarine and coastal systems, uses a range of common coastal organisms in which the Green crab is included (*Galloway et al.*, 2004a, 2006). Biomarkers have been successfully applied within the context of the WFD risk assessment process, usually to reduce uncertainty and to provide evidence of the existing impacts, together with
chemical and ecological monitoring (e.g., Hagger et al., 2008, 2009). Moreover, the usefulness of biomarkers to monitor the effectiveness of remediation treatments was highlighted by Depledge et al. (1995). In a more holistic assessment, the indices IBR and BRI, which emerged from the biomarker approach, were both considered as valuable tools to integrate the responses of C. maenas regarding the differently impacted areas. Finally, a classification scale was developed based on the biomarker approach and used to discriminate from slight to severe alterations from C. maenas's normal responses, aiming to describe the potential toxicity of pollutants to crabs (Hagger et al., 2009). Therefore, it is possible to conclude that a wide range of tools based on the biomarker approach was developed to achieve highly relevant toxicity results using C. maenas as a test organism, especially as regards the assessment of sublethal effects. Thus, the present review highlights the pivotal role of the macroinvertebrate C. maenas as a test organism in ecotoxicological relevant laboratory studies and as a suitable bioindicator of aquatic environmental health. Therefore, we conclude that C. maenas is a suitable model organism in ecotoxicology research and suggest the biomarker approach applied to C. maenas as a line of evidence in standardised environmental quality assessment. Moreover, this crab fulfils many of the criteria of the protocol for selection of sentinel species and collection of specimens (EROCIPS, 2006), is very easy to catch from wild populations and is also easy to maintain in the laboratory. However, since the final expression of high quality toxicity testing is a reliable result, the outcomes from the present review recommend gender, size and morphotype separation in C. maenas experimental designs and data evaluation. Moreover, nutritional status should be taken into account, especially in long-term studies. Studies should also consider the resistance ability of crabs to face historical and concurrent contamination. Finally, experimental temperature and salinity should be harmonised for reliable comparisons between studies.
Even though the application of biomarkers on *Carcinus* sp. has been vastly studied and has provided innumerable advantages, specificity is a major concern, as the outcomes gathered in the present review exposed the difficulty to link biomarker responses to a specific type of pollution. Therefore, a complete *C. maenas* genome sequencing programme is essential for cutting-edge research. The rapid growth of molecular biology and the development of laboratory technology should improve the sensitivity and specificity of the biomarker approach in order to accurately diagnose environmental stress. Moreover, it is crucial that toxicological studies and chemical analyses are carried out simultaneously so as to enable accurate links between biomarker response and pollutants. Also, further research on the measurement of biomarkers in multiple tissues is needed to gain understanding on toxicokinetic aspects of pollutant exposure. Concerning future topics of research, the development of *in vitro* bioassays for crabs is of major importance, and mitochondrial toxicity assessment and cell-based assays are considered key areas to be further developed. Mitochondria are of particular interest because of their pivotal role in organisms’ energy metabolism, and similar to what happens in the development of new drugs, mitochondrial toxicity assessment can be used as a tier 1 testing approach. Concerning cell lines derived from crabs, it remains a challenge for research development.

Since animal protection legislation tends to exclude the use of vertebrates (e.g., fish, amphibians) and invertebrate cephalopods from, and moderate the use of decapods in scientific research and risk assessment of aquatic environments, the present review found that crab urine (sampling procedure described by Bamber and Naylor (1997)) and Hm are suitable matrices for chemical analyses and biomarker determination, as sampling techniques are considered non-destructive. Since crab urine is relatively free from lipids and proteins, samples were considered suitable for monitoring PAH metabolites (Fillmann et al., 2002; Galloway, 2006). Moreover,
Dissanayake and Bamber (2010) highlighted the use of fluorescence spectrophotometry as a rapid and cost-effective technique to identify PAH metabolites in *C. maenas's* urine samples as a result of PAH exposure. Concerning the use of Hm samples to detect adverse effects, lysosomal membrane stability, a cellular endpoint, was considered a sensitive tool for evaluating exposure to sublethal concentrations of pharmaceutical drugs and PAHs under laboratory conditions by Aguirre-Martínez et al. (2013a) and Dissanayake et al. (2008a, 2010). The authors also concluded that these techniques provide a robust tier 1 testing approach for the rapid assessment of marine pollution. Furthermore, technological developments have facilitated the non-invasive monitoring of *C. maenas's* responses, namely cardiac (heart rate), respiratory, and locomotion activities, which were considered important physiological and behavioural endpoints (Aagaard et al., 1991). This approach allows simultaneous (cardiac, respiratory and locomotor activities) long-term recordings with a minimal disturbance of experimental organisms (e.g., Styrishave et al., 1999; Styrishave et al., 2003). Also, *C. maenas* appears to be a suitable candidate to assess behaviour at the individual level in a marine context by means of the automated method Multispecies Freshwater Biomonitor™, as was demonstrated by Stewart et al. (2010). To conclude, the present literature survey reports the suitability of several important non-destructive endpoints covering different biological levels which can be successfully used in toxicity testing with *C. maenas*, as well as their compliance with the prevailing ethical concerns.

Finally, estuarine environments are currently considered as highly valuable and their health and conservation status are seen as a priority. However, its complexity requires the measurement and integration of a great quantity of information on biology and ecology, as well as toxicological data. Therefore, based on the current scientific knowledge regarding the biology and ecology of *C. maenas* and the
extensive studies in toxicology found for the present review, we acknowledge the crab *C. maenas* as a reliable test organism for routine ecotoxicity testing, especially in what concerns the application of the biomarker approach. Additionally, we believe that this gathering of knowledge and data will enable the advancement of our understanding of the risks for the estuarine environment, so as to contribute with constructive recommendations for environmental management strategies and policy decision-making in order to prevent environmental hazard and, ultimately, human risk.
**Biochemical and Physiological Responses of Carcinus maenas to Temperature and the Fungicide Azoxystrobin**

As showed on the previous study, increasing evidence has been found for the pivotal role of the crab *C. maenas* in the assessment of the impact of pollutants, especially in the ecotoxicological assessment of coastal and transitional waters, and as a valuable alternative for vertebrate use, which reflect ethical concerns. Therefore, an ecologically relevant study was conducted to evaluate the biochemical (SOD and GST activities) and physiological (oxygen consumption rates) responses of *C. maenas* to an environmental concentration of azoxystrobin (30 µg L\(^{-1}\), see Rodrigues et al., 2013c) in the context of climate change projections (extreme temperatures). The antioxidant enzyme SOD was chosen since in his review concerning oxidative stress in aquatic organisms Lushchak (2011) reports an enhancement of oxygen consumption, and then a possible increase of ROS production as a result of temperature rise. Moreover, azoxystrobin may also be able to produce ROS, since the generation of \(\text{H}_2\text{O}_2\) by the pesticide myxothiazol, which has the same mode of action as azoxystrobin, was demonstrated by Starkov and Fiskum (2001) in rat heart and brain mitochondria. GST was chosen since was considered a key enzyme of *phase II* detoxification metabolism, which can also directly detoxify free radicals (this project, Rodrigues and Pardal, 2014). All the endpoints selected were measured using the mitochondrial fraction isolated from the crabs’ hepatopancreas. Mitochondrial fraction choice was based on the fact that mitochondria is a recognised experimental model in many areas of the biomedical sciences, and the fact that, among others, it plays a well-known crucial contribution
for organisms’ metabolism and cellular energy status (e.g., Smith et al., 2012). Besides SOD and GST enzymatic activities and oxygen consumption rates, parameters such as mitochondrial protein content per gram of fresh hepatopancreas and the Coupling Index, which relates the mitochondrial oxidative capacity with cellular energy production, were also determined. In this study, three experimental temperatures were tested (5°C, 22°C and 27°C). The control temperature of 22°C was established by the guideline ASTM E729 (2002), and extremes temperatures (5°C and 27°C) were chosen based on the study of crabs’ tolerance to environmental temperature according to the surveyed literature. The hypothesis proposed that temperature and azoxystrobin would affect the biochemical and physiological responses of C. maenas, as well as that extreme temperatures (5°C and 27°C) would promote azoxystrobin toxicity by unbalancing the natural capability of crabs to handle a single stressor. The present study also had the intent to verify if the mitochondrial toxicity assessment can be used as a tier 1 testing approach when applied to invertebrates, thus offering ethical advantages, as highlighted by Rodrigues and Pardal (2014).

Abstract

Studies on the effects of thermal stress are currently becoming increasingly pertinent as climate change is expected to cause more severe climate-driven events. Hence, in order to overcome some of the above-mentioned gaps which might be related to this phenomenon, an ecologically relevant study was conducted to evaluate the biochemical and physiological responses of C. maenas to temperature and azoxystrobin. Crabs’ responses were assessed after a 10-d acclimation at different temperatures (5°C, 22°C and 27°C) of which the last 72 hours were of exposure to an environmental concentration of azoxystrobin. Superoxide dismutase (SOD) and glutathione S-transferase (GST) activities,
mitochondrial oxygen consumption rates and protein content, as well as the Coupling Index were determined. Results showed statistically significant different effects of SOD and all oxygen rates measured promoted by temperature, and that neither 30 µg L⁻¹ of azoxystrobin nor the combined effect were crab-responsive. Protein content at 5ºC was statistically higher when compared with the control temperature (22ºC). The Coupling Index revealed both a slight and a drastic decrease of this index promoted by 5ºC and 27ºC, respectively. Regarding azoxystrobin effects, at 22ºC, this index only decreased slightly. However, at extreme temperatures it fell 47% at 5ºC and slightly increased at 27ºC. Results provided evidence that crabs’ responses to cope with low temperatures were more effective than their responses to cope with high temperatures, which are expected in future climate projections. Moreover, crabs are capable of handling environmental concentrations of azoxystrobin. However, the Coupling Index showed that combined stress factors unbalance crabs’ natural capability to handle a single stressor.

***

Materials and methods

Experimental animals

As in Portugal the Guadiana estuary was considered by Vasconcelos et al. (2007) the estuary least affected by agriculture, C. maenas crabs were collected from wild populations in this estuary, in the Castro Marim Salt Marsh Nature Reserve (37º 13.099' N, 7º 25.968' W) using baited circular drop nets. Collection was carried out in three different batches during 2013, in January, February and April. To avoid behavioural and physiological variability associated with crab gender, size and morphotype, only intermoult male green colour-form individuals of uniform size (4-5 cm carapace width) were selected for the present study. Organisms presenting
signs of being infected by the parasitic barnacle *Sacculina carcini* were discarded. As required in *ASTM E729 (2002)* guideline, each batch of organisms was acclimated in a temperature-controlled room (22°C) for at least seven days. A sufficient acclimation period is of great importance to ensure that the different batches of crabs are free of the stress triggered by environmental conditions prior to experimentation, and in most studies 7-d acclimations were carried out for *C. maenas* (this project, Rodrigues and Pardal, 2014). During acclimation, crabs were maintained in aerated recirculating aquatic systems composed by glass tanks (50 × 35 × 25 cm) and appropriate life support systems. Each tank had 20 L of natural seawater (salinity progressively adjusted to 34) and approximately 25 organisms. Two shelters of PVC pipes were supplied to each tank as environmental enrichment. Crabs were fed every other day with the saltwater clam *Paphia undulata* purchased in a local food shop and traded by Gelpeixe (Portugal). Nevertheless, all crabs were starved for the 24 hours prior to use, thus ensuring that all animals were at a similar starting point. Physico-chemical acclimation conditions such as water salinity, pH and dissolved oxygen were measured daily using WTW probes.

**Analytical standards and solutions**

An azoxystrobin stock standard solution (360 mg L⁻¹) was prepared in acetone p.a. (pro analysis) and stored at -18°C. Exposure media was prepared freshly on the day of use in reconstituted water with a salinity adjusted to 34 (tropic marin salt).

**Experimental design**

Three tested experimental temperatures (5°C, 22°C (control, *ASTM E729 (2002)*) and 27°C) were performed separately in time on independent batches of organisms (5°C: crabs collected in January; 22°C: crabs collected in February; 27°C: crabs
collected in April). Experimental temperatures took place in a constant-temperature chamber with illumination programmed to 16 h light/8 h dark photoperiod (ASTM E729, 2002) (Binder KBW 400). For each experimental temperature, three different aerated renewal treatments were developed during the 10 days of the experiment, namely: control - C, solvent control - SC, and azoxystrobin - AZX. Air filtration was done through a 0.2 µm syringe filter (Minisart, Sartorius Stedim Biotech) and air was carried by PTFE tubing (Bola S1810-18). Each experimental treatment (C, SC and AZX) was repeated twice, each considered an independent experimental replicate (N = 2), and referred to hereafter as experimental tests (Fig. 17). As only one mitochondrial isolation can be performed per day, experimental tests started in consecutive days. To begin each experimental test (day 0), a set of 12 crabs were randomly selected from the stock tanks and transferred to individual experimental capped glass flasks (9 cm Ø) with 500 mL of reconstituted water with a salinity adjusted to 34. Weight and carapace width were previously registered. Water was checked daily for evaporation and ultrapure water purified with a Milli-Q Biocel System was added whenever necessary. Crabs were fed on days 3 and 6, and the water exchange was carried out on days 4 and 7. Physico-chemical experimental conditions such as water salinity, pH and dissolved oxygen were measured whenever water was changed using WTW probes. Visible inspection of the crabs were performed every day and the organisms which moulted during the experiments were discarded as they alter their metabolism during the mouling process and, thereby, their response to the tested parameters. Organisms which died were also discarded.
On day 7 of all experimental temperatures, 35 μL of acetone were added to water exchange in solvent control experimental tests (SC), and in azoxystrobin experimental tests (AZX) water was replaced by azoxystrobin exposure media (nominal concentration of 25 μg L⁻¹) (Fig. 17). In all AZX, water samples were taken at the end of the test to verify by a validated confirmatory method the concentration of azoxystrobin used.

Analytical methodology

Azoxystrobin analyses in water samples were carried out in the laboratory of the Instituto Superior Técnico (University of Lisbon, Portugal). Analyses were performed using 1.0 μL of sample in dichloromethane by liquid-liquid extraction methodology. The separation and quantification of azoxystrobin was done by GC-MS. A Restek TG-5MS column, 30 m × 0.25 mm, 0.25 μm (Supelco) was employed using helium as a carrier gas at a 1.0 mL min⁻¹ flow rate. The temperature of the injector was kept at 250°C. The oven temperature was as follows: 230°C at 20°C min⁻¹ held for 1 min., then 310°C at 25°C min⁻¹ and held for 6 min. Mass detector conditions were: 310°C as transfer line temperature and 250°C as ion source temperature. Selected Ion Monitoring (SIM) mode was chosen and several specific ions were selected:
329, 344, 345, 372, 388, 403. (in bold, ion used for quantification). The limit of the quantification method was 13 µg L⁻¹.

The azoxystrobin concentration was attained by calculating the geometric mean of the initial nominal (25 µg L⁻¹) and the final measured concentrations of each AZX experimental test, as recommended by Traas (2001). Hence, 30 µg L⁻¹ is considered the concentration of azoxystrobin used in the present work.

Mitochondrial isolation

Purified mitochondria were isolated from hepatopancreatic tissue of freshly killed C. maenas previously anaesthetised on ice and then euthanised by destruction of the ventral ganglion. All organisms presenting signs of moult initiation or of parasite infection at the moment of dissection were discarded. In each experimental test, hepatopancreas were pooled to ensure an adequate quantity of mitochondria, and the total weight was registered. Also, visual inspection of crabs’ hepatopancreas consistency was performed in order to check possible differences by experimental temperature. All the manipulations were carried out at ice-cold temperatures with the exception of centrifugations, which were performed at 4°C. Mitochondria purification followed the method outlined by Chen and Lehninger (1973) with slight modifications which are briefly described below. Firstly, hepatopancreatic tissue was washed and then immersed in homogenization medium (300 mM mannitol, 83 mM sucrose, 10 mM EDTA, 5 mM Tris/chloride, and 1% bovine serum albumin (BSA), at pH 7.4). The homogenate was achieved by 5-10 passes (<500 rpm) in a pre-cooled glass/Teflon homogeniser. Mitochondria were then isolated by conventional differential centrifugations (Sigma 3-16PK) as follow: at 700g for 12 min., and the supernatant collected and centrifuged again at 10,000g for 12 min. to sediment the mitochondria. The resulting pellet was resuspended in washing medium (300 mM mannitol, 83 mM sucrose, 5 mM Tris/chloride, and 0.2% BSA, at pH 7.4) and
centrifuged twice at 10,000g for 12 min. Finally, the mitochondrial pellet was resuspended in washing medium and the final volume was registered for further determination of the content of mitochondria protein per gram of fresh hepatopancreatic tissue. The mitochondrial protein concentration (mg mL⁻¹) was determined colorimetrically using biuret-reagent (Gornall et al., 1949) and BSA as standard. A correction was made, as final mitochondrial suspension had 0.2% of BSA.

Mitochondrial respiratory endpoints

Since preliminary studies showed that mitochondria obtained from C. maenas hepatopancreas were loosely coupled, and the most effective respiratory substrate was mixture glutamate/malate (G/M), the contribution of the mitochondrial complex I (NADH-dependent oxidation pathway) was chosen to investigate the physiological effects of temperature and azoxystrobin in the present study. Thereby, immediately after mitochondrial isolation, mitochondrial state 2 respiration was evaluated using G/M (final concentration 10 mM/5 mM); and then the maximum ADP-stimulation (state 3) and the state FCCP were obtained by sequential additions of ADP (final concentration 1 mM) and carbonylcyanide p-trifluoromethoxyphenyl-hydrazone (FCCP, final concentration 1 µM). To allow the exhaustion of endogenous substrates, a 3-min. equilibration time was performed before mitochondria (1.0 mg) are energized with G/M. The maximum ADP-stimulated respiration determined was considered a measure of mitochondrial oxidative phosphorylation capacity and the maximum electron transport capacity was quantified as oxygen consumption in non-coupled mitochondria, induced by the addition of FCCP. Thus, in the present study, the ratio between maximum ADP-stimulated respiration and oxygen consumption in the presence of the substrate (state 2) was called Coupling Index. When a value of this index is close to one, it indicates that mitochondrial proton pumping into the
intermembrane space becomes functionally uncoupled from ATP synthesis. At the end of the assays, the remaining mitochondrial suspension was deep-frozen (-80°C) for further enzyme activity determinations.

Mitochondrial oxygen depletion was measured in a 22°C thermostated glass chamber with stirring and a glass stopper using a Clark oxygen probe (Yellow Springs Instruments) (Estabrook, 1967). The signal of the probe was directed via a control unit (YSI 5300) to a Kipp & Zonen recorder. For each measurement, the chamber was filled with 0.85 mL of respiration medium (200 mM mannitol, 83 mM sucrose, 10 mM potassium dihydrogen phosphate, 10 mM magnesium chloride and 10 mM Tris/chloride, at pH 7.2). Calibration of the latter system was performed every day of use at measurement temperature (22°C) and using respiration medium. All assays were run at least in duplicate. For the final calculations of mitochondrial respiratory activity, oxygen solubility (270.94 μM) was accessed at http://www.colby.edu/chemistry/CH331/O2%20Solubility.html from the values of temperature (22°C) and salinity (1.4) of the respiration medium. The rates of oxygen consumption were calculated from the recorder tracing and expressed as nmol O₂ min⁻¹ mg protein⁻¹.

Mitochondrial SOD and GST

Non-specific SOD activity was measured by its ability to inhibit superoxide radical dependent reactions according to the procedure described by Peskin and Winterbourn (2000) and using the SOD assay kit- WST (Dojindo S311). SOD activity was determined by a kinetic method according to the procedure described in the manual provided by the supplier and using the final concentration of 0.15 mg mL⁻¹ of mitochondrial protein. SOD results, inhibition rate in percentage, were calculated also following the protocol provided by the supplier. GST activity was determined according to the procedure described by Habig et al. (1974) and
adapted to microplate using the final concentration of 0.6 mg mL\(^{-1}\) of mitochondrial protein and 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. GST results (nmol min\(^{-1}\) mg protein\(^{-1}\)) were blanked using the arithmetic mean absorbance of six buffer blanks (negative controls) in the same microplate and a correction for light path length was performed, and results were determined considering that CDNB conjugate at 340 nm have an extinction coefficient of \(\varepsilon = 9.6\, \text{mM}^{-1}\, \text{cm}^{-1}\). SOD and GST enzyme activities were determined in 96-well microplates (SOD: Brand 781602, GST: Costar 3635) using a Multi-Mode Microplate Reader (Synergy HT, BioTek). Duplicate tests were run on different microplates, but the same reaction buffer solution was used in both tests. The coefficient of variation of the mean (CV, in percentage) of the negative controls was calculated for each test/plate in order to ascertain reproducibility and was used as a test acceptance criterion. CV was calculated by the equation:

\[
CV = \frac{SD}{\sqrt{N}}/\text{arithmetic mean} \times 100
\]

where SD is the standard deviation and \(N\) the number of negative control wells per test/plate (present study, \(N = 6\)). The acceptance criterion is that the CV of each test/plate be less than or equal to 20%.

Statistical analysis

The values of the content of mitochondrial protein per gram of fresh hepatopancreas of control experimental treatments were subjected to a one-way ANOVA, followed by a post hoc Dunnett’s test, to verify significant differences by experimental temperature. One-way ANOVA was also used to test significant differences between control and solvent control experimental treatments for all determined endpoints (Zar, 2010). Due to logistical constrains, experimental temperatures were conducted at different times. Nevertheless, a two-way ANOVA was used to verify significant differences using temperature and azoxystrobin as factors. This approach was
undertaken based on the assumption that by collecting all model organisms at exactly the same site within a period as short as four months and conducting a 7-d acclimation period, the across-time and baseline inter-individual variability of responses were similar. This analysis was followed by a post hoc Dunnett’s test to further evaluate temperature effects, as neither temperature nor the interaction between temperature and azoxystrobin were significant. The experimental temperature of 22ºC was considered as temperature control (ASTM E729, 2002). ANOVAs were used after testing the assumption of homogeneity of variances by the Bartlett’s test. A value equal or inferior to 0.05 was considered as level of statistical significance (P). Statistical analyses were performed using the STATISTICA 7.0 software.

Results

Biological and physico-chemical parameters

A summary of the biological and physico-chemical parameters by experimental temperature is presented in Table 23. Crabs’ behaviour under the different ambient temperatures was observed during the 10-d experimental tests. For instance, crabs under the control experimental temperature (22ºC) were found to be calm, a behaviour similar to that observed during the acclimation period. To cope with the 5ºC temperature, crabs became dormant. On the other hand, at 27ºC, crabs showed signs of some agitation, kneading the oxygenation capillaries with the clamps. Also, organisms which moulted on feeding day or the day after food was supplied did not eat, showing that feeding declines or ceases during the moulting process. The content of mitochondrial protein per gram of fresh hepatopancreatic tissue at 5ºC was more than fivefold higher when compared with the results of control temperature (22ºC) treatments, which was statistically significant (P <0.001). Mitochondrial membrane fluidity was not assessed in the present study. However,
hepatopancreatic consistency differences by experimental temperatures were observed during the dissection process, showing decreased firmness as temperature raised.

Table 23 Biological and physico-chemical parameters (mean ± SD) by experimental temperature.

<table>
<thead>
<tr>
<th>Units</th>
<th>5ºC</th>
<th>22ºC</th>
<th>27ºC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td>g</td>
<td>17 ± 4, N=72</td>
<td>18 ± 3, N=72</td>
</tr>
<tr>
<td>Carapace width</td>
<td>mm</td>
<td>43 ± 3, N=72</td>
<td>44 ± 3, N=72</td>
</tr>
<tr>
<td>Initial dissolved oxygen</td>
<td>%</td>
<td>97 ± 3, N=199</td>
<td>99 ± 3, N=206</td>
</tr>
<tr>
<td>Final dissolved oxygen</td>
<td>%</td>
<td>95 ± 15, N=195</td>
<td>80 ± 14, N=188</td>
</tr>
<tr>
<td>Initial pH</td>
<td></td>
<td>8.0 ± 0.2, N=187</td>
<td>7.9 ± 0.1, N=206</td>
</tr>
<tr>
<td>Final pH</td>
<td></td>
<td>7.5 ± 0.1, N=179</td>
<td>7.4 ± 0.2, N=199</td>
</tr>
<tr>
<td>Percentage of crabs that did not eatb</td>
<td>%</td>
<td>68.8</td>
<td>0</td>
</tr>
<tr>
<td>Percentage of moulted crabsc</td>
<td>%</td>
<td>1.4</td>
<td>12.5</td>
</tr>
<tr>
<td>Percentage of dead crabs</td>
<td>%</td>
<td>2.8</td>
<td>1.4</td>
</tr>
<tr>
<td>Mitochondrial protein content per gram of hepatopancreas</td>
<td>mg protein g⁻¹</td>
<td>4.3 ± 1.2, N=5</td>
<td>0.8 ± 0.1, N=5</td>
</tr>
</tbody>
</table>

Table 23 Biological and physico-chemical parameters (mean ± SD) by experimental temperature.

Mitochondrial SOD and GST

The laboratory enzymatic tests performed were all accepted since the highest CV calculated was 12.7%. Therefore, a mean value of the duplicates was considered for ANOVA tests. Crabs' biochemical responses showed that no significant differences between control and solvent control treatments were found in any of the experimental temperatures in both enzymes assessed (SOD: P ≥0.388 and GST: P ≥0.125). Hence, from now on, the present study only reports control and azoxystrobin treatment results (Table 24). The azoxystrobin environmental concentration tested (30 µg L⁻¹) and the interaction between temperature and this fungicide did not reveal statistically significant effects on enzymes (Table 25). Moreover, GST data also exhibited no significant effects concerning temperature. However, SOD outcomes (inhibition rate %) showed significant effects of temperature, indicating a significant decrease (Dunnett's test, P = 0.001) at 5ºC (mean value of 51% inhibition), when compared with the control temperature of 22ºC (mean value of 87% inhibition).
Table 24 Biochemical results (mean ± SDa) of control (C) and azoxystrobin (AZX) treatments by experimental temperature.

<table>
<thead>
<tr>
<th>Units</th>
<th>5°C</th>
<th>22°C</th>
<th>27°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD inhibition rate %</td>
<td>51 ± 0</td>
<td>64 ± 17</td>
<td>87 ± 3</td>
</tr>
<tr>
<td>GST nmol min⁻¹ mg protein⁻¹</td>
<td>10.4 ± 0.9</td>
<td>11.3 ± 0.8</td>
<td>7.5 ± 3.4</td>
</tr>
</tbody>
</table>

Table 25 Summary of the two-way ANOVA results applied to all the metabolic rates determined. Bold indicates significance.

<table>
<thead>
<tr>
<th></th>
<th>T</th>
<th>AZX</th>
<th>T+AZX</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD</td>
<td>F (2) = 22.76, P = 0.002</td>
<td>F (1) = 0.92, P = 0.374</td>
<td>F (2) = 0.94, P = 0.442</td>
</tr>
<tr>
<td>GST</td>
<td>F (2) = 4.92, P = 0.054</td>
<td>F (1) = 0.81, P = 0.403</td>
<td>F (2) = 1.30, P = 0.340</td>
</tr>
<tr>
<td>G/M (state 3)</td>
<td>F (2) = 22.30, P = 0.002</td>
<td>F (1) = 0.58, P = 0.474</td>
<td>F (2) = 1.81, P = 0.243</td>
</tr>
<tr>
<td>ADP (state 3)</td>
<td>F (2) = 10.46, P = 0.011</td>
<td>F (1) = 0.24, P = 0.639</td>
<td>F (2) = 0.09, P = 0.920</td>
</tr>
<tr>
<td>FCCP (state FCCP)</td>
<td>F (2) = 24.41, P = 0.001</td>
<td>F (1) = 3.63, P = 0.106</td>
<td>F (2) = 1.09, P = 0.395</td>
</tr>
</tbody>
</table>

Mitochondrial respiratory endpoints

Results of physiological responses, which correspond to respiratory rates determined after the addition of G/M, ADP and FCCP, showed no statistically significant differences between control and solvent control treatments (P ≥0.326) in any of the experimental temperatures. Hence, from now on, only control and azoxystrobin treatments results are reported. As shown in Fig. 18, a consecutive and expected pattern of oxygen consumption increase can be observed in control treatment (C) at control temperature (22°C) after the sequential addition of G/M, ADP and FCCP. At 22°C, the addition of ADP to mitochondria doubled the respiration rate, yielding clearly distinguishable state 3 respiration (ADP-stimulated respiration), and the maximum respiration rate was achieved after the addition of FCCP. The mentioned pattern was repeated at 5°C (C treatment) but with lower oxygen consumption rates. However, at 27°C, control treatment reflects the absence of the same pattern, showing an enhancement of respiration rates after the addition of G/M and ADP similar to that quantified as the maximum capacity. Concerning azoxystrobin treatment (AZX), at the control temperature of 22°C the mentioned pattern remains, but displaying slightly lower rates. However, the association of azoxystrobin with low temperature results in alterations to the pattern, and when
azoxystrobin was associated with high temperature (27ºC) the pattern was no longer expected, since at 27ºC the absence of the pattern was associated with high temperature (see C at 27ºC, Fig. 18). Two-way ANOVA results showed that only temperature statistically influenced mitochondrial respiration rates (Table 25). In what concerns control treatments at all experimental temperatures, and as is shown in Fig. 18, a statistically inhibitory effect on mitochondrial respiration is observed at 5ºC, after the addition of G/M (inhibition of 65%, Dunnett’s test: P <0.05), ADP (inhibition of 71%, Dunnett’s test: P <0.01) and FCCP (inhibition of 79%, Dunnett’s test: P <0.001); with a maximum electron transport capacity (after adding FCCP) similar to that produced by G/M at the control temperature (22ºC). When compared to control temperature, mitochondrial respiration after being energised increased by 85% at 27ºC, which was significantly higher (Dunnett’s test, P <0.01), while maximum electron transport capacity was statistically decreased (Dunnett's test, P <0.01).

Fig. 18 Oxygen consumption rates (mean ± SD) of mitochondria from the hepatopancreas of Carcinus maenas under different conditions of temperature and 30 µg L⁻¹ of azoxystrobin. * (P <0.05) and ** (P <0.001), both denote means significantly different from those obtained at the control temperature of 22ºC, independently of the azoxystrobin concentration (by Dunnett’s test).
**Coupling Index**

Coupling Index results were plotted in Fig. 19. *C. maenas* maximum coupled mitochondria were obtained at the control temperature of 22ºC, and a slight and an abrupt decrease were observed under control physiological conditions at 5ºC and 27ºC, respectively. At extreme temperatures, the tested azoxystrobin environmental concentration seems to highlight uncoupling/coupling effects, since at 5ºC this index abruptly decreased, and at 27ºC it slightly increased.

![Fig. 19 Coupling Index results.](image)

**Discussion**

Aquatic organisms are invariably exposed to multiple environmental stressors. It is, therefore, essential that we improve our knowledge on the effects of such complex scenarios. The present study was undertaken to determine the biochemical and physiological responses of *C. maenas*, a recognised estuarine model organism, to temperature and the most widely applied fungicide, azoxystrobin.

In order to support the results gathered in the present study, comparisons with data and trends from similar studies were attempted. For instance, temperature dependence of SOD activity was established by the outcomes of the present study, suggesting that, in crabs, the liver mitochondria could deal with the increase in superoxide anion radicals promoted by low temperatures. A similar result was also found by Pavlović and collaborators, since Mn-SOD activity was statistically higher.
in samples collected in winter in two locations of the Southern Adriatic Sea (Pavlović et al., 2010). The same tendency was observed by Monari et al. (2007) in the clam Chamelea gallina with a significant decrease in haemocyte Mn-SOD and Cu/Zn-SOD activities with temperature rise. Results of the present study concerning control values of GST activity obtained at the experimental temperature of 22°C (mean ± SD, 7.5 ± 3.4 nmol min⁻¹ mg protein⁻¹) were in line with those obtained from C. maenas hepatopancreas in controls carried out by other authors, e.g., ≈1 nmol min⁻¹ mg protein⁻¹ (Mesquita et al., 2011), ≈2 nmol min⁻¹ mg protein⁻¹ (Aguirre-Martínez et al., 2013c), and ≈12 nmol min⁻¹ mg protein⁻¹ (Rodrigues et al., 2013b). Also, the influence of temperature on the crabs’ GST activity is in agreement with the results obtained by Bebianno et al. (2007), since these authors reported the same outcome after measuring GST activity in the M. galloprovincialis post-mitochondrial fraction of gills and digestive gland tissue at different temperatures. Concerning physiological results, the mitochondrial oxygen consumption after G/M activation of C. maenas’s hepatopancreas obtained from the control experimental treatment at 22°C nearly doubled the G/M oxidation rate attained by Nesci et al. (2011) from the digestive gland of M. galloprovincialis mitochondria, and was about the triple of that obtained by Völkel and Grieshaber (1997) in the mitochondria of the lugworm Arenicola marina (body-wall tissue), after using a saturated concentration of malate, thus suggesting a considerable variation among species. No studies were found with crustaceans and similar mitochondrial assays which would enable the comparison of our results. Total protein (mg g⁻¹ wet mass) in the liver of the red mullet (Mullus barbatus) from the Near Bar and Estuary of the River Bojana, both located in the Southern Adriatic Sea, was determined in winter and spring. Corroborating our results, a higher statistically significant result was obtained in the samples collected in winter at both sampling locations (Pavlović et al., 2010), indicating the importance of increase liver protein content to cope with low water temperatures.
A clear outcome from the present study is that thermal stress induces alterations in both oxidative defence, and cellular respiration and phosphorylation of aquatic organisms, even in eurythermal ones. It is known that the evolutionary adaptation of ectothermal species to low temperatures requires increased mitochondrial densities to compensate for the reduced mitochondrial performance in cold environments (e.g., Guderley, 2004). Results from the present study corroborate this, as they showed significant differences in the content of mitochondrial protein by experimental temperature, indicating a significant rise at the low temperature of 5°C. Nevertheless, a drastic reduction in the rates of mitochondrial oxygen consumption was observed, possibly explaining why crabs entered into metabolic depression at 5°C. On the other hand, results showed that antioxidant enzymes as SOD may regulate oxidative stress at low temperatures. Moreover, the effect of thermal stress in biological membranes are vastly covered in literature (e.g., Crockett et al., 2001; van Dooremalen et al., 2011; Pernet et al., 2007), as a decrease in temperature usually reduces membrane fluidity, as observed in the present study. This could explain the observed maintenance of the functionality of mitochondria at the extreme temperature of 5°C. On the other hand, exposure to high temperatures caused a decrease in hepatopancreatic tissue consistency (qualitatively observed), thus possibly potentiating proton leakage. This was reflected in the results of oxygen consumption rates and Coupling Index at 27°C, hence showing an uncoupling situation (Figs. 18 and 19). The respiratory chain energised by G/M almost doubled its work to face the proton leakage, i.e., the flow of protons across the inner mitochondrial membrane, promoted by the rise of the temperature, and state FCCP reveals that the respiratory chain may possibly be affected at 27°C, since a higher value was expected after FCCP was added (Fig. 18). It is known that the coupling state of mitochondria is a key component of oxidative phosphorylation and that a disruption to this process by temperature could contribute to a range of pathologies, which were reviewed by Fosslien (2001). Moreover, exposure to high temperatures
causes the inhibition of the anti-oxidant enzyme SOD. Also, the Coupling Index determined in the present study revealed a drastic decrease of this index promoted by the high temperature of 27°C (Fig. 19). Based on mortality records, and compared to the control temperature, a twofold increase was observed at the low temperature of 5°C, and at 27°C mortality was fourfold higher. Therefore, these results corroborate the role of temperature as an important environmental factor in aquatic ecosystems for it influences biological activity.

The addition of a classic uncoupling agent such as FCCP leads to a permanently high respiration rate for it carries protons across the inner mitochondrial membrane, thus dissipating the electrochemical gradient that drives ATP synthesis. Thereby, mitochondria need to increase the electron flow and thus oxygen consumption in order to maintain membrane potential. After the addition of FCCP, the present study reported a mean value of 73.9 nmol O₂ min⁻¹ mg protein⁻¹ in the control experimental treatment at the optimal experimental temperature of 22°C (Fig. 18). Therefore, a similar value was expected after the addition of substrate to the control of the experimental treatment at the high temperature of 27°C, since an uncoupling effect due to temperature was observed. However, at 27°C, control respiratory rates only presented a mean value of 31.5 nmol O₂ min⁻¹ mg protein⁻¹. This value suggests that this eurythermic invertebrate develops a defence mechanism to slow down respiratory rates so as to cope with high temperatures.

Results gathered from the present study reveal that C. maenas showed some degree of tolerance to azoxystrobin exposure. However, the environmental concentration used (30 µg L⁻¹) and the high variability associated with the low number of replicates (N = 2) in AZX treatments, displayed in Fig. 18, could explain the absence of azoxystrobin statistically significant results. Nevertheless, results from the Coupling Index pointed out a tendency that is worth noticing. For instance, the uncoupling effect of azoxystrobin at 5°C was evident, for this index showed a
decrease of 47% in AZX results at this low temperature (Fig. 19). This index’s slight increase with azoxystrobin at 27°C could be explained by the fact that mitochondria are uncoupled as a result of temperature and, therefore, the added azoxystrobin could have binded in some way to membranes, slightly increasing this index.

From the ecological standpoint, understanding temperature effects on ectothermal aquatic organisms is of paramount importance, especially in the current context of global change scenarios, e.g., increase of Earth’s average temperature, increase of extreme climate-driven events. Results gathered by the present study provide evidence that mitochondrial functionality may limit species survival and potential future distributions since crabs’ biochemical and physiological responses to low temperatures were more effective than those which were put in motion to cope with high temperatures. Moreover, the present study concludes that crabs are capable of handling environmental concentrations of azoxystrobin. However, results from the Coupling Index pointed out a tendency that is worth noticing, showing that combined stress factors unbalance the natural capability of crabs to handle a single stressor. However, further research is necessary to clarify this interaction (e.g., increasing the azoxystrobin concentration).

Findings from the present study suggest that hepatopancreatic enzymatic activity and mitochondrial performance are sensitive indicators of thermal stress in *C. maenas*. However, concerning the use of the mitochondrial toxicity assessment as a tier 1 testing approach to be routinely applied on *C. maenas*, even though promising, it still requires several further studies. For instance, a large set of pesticides and other environmental pollutants with different modes of action and covering several orders of magnitude of Kow should be tested. Nevertheless, and in line with previous conclusions (this project, Rodrigues and Pardal, 2014), this invertebrate was considered as a reliable test organism, thus being an alternative to the use of vertebrates in aquatic ecotoxicity testing, which supports ethical
concerns. Finally, this study reinforces the importance of applying the standard temperatures indicated by guidelines in ecotoxicology research to ensure inter-study comparability.
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Chapter IV – Azoxystrobin effects at the cellular level


Cardiomyocyte H9c2 cells present a valuable alternative to fish lethal testing for azoxystrobin. *Environmental Pollution* 206, 619-626.

doi 10.1016/j.envpol.2015.08.026

4.143 IF_{2014} (0 citations, web of knowledge), Q1_{2014} Pollution
CARDIOMYOCYTE H9c2 CELLS PRESENT A VALUABLE ALTERNATIVE TO FISH LETHAL TESTING FOR AZOXYSTROBIN

The present Chapter proposes to compare the growth inhibition potential of azoxystrobin on mammalian (A549, HepG2, BJ and H9c2) and fish (VSa16 and ABSa15) cell lines, with the juvenile S. aurata short-term lethal test. Outcomes of the present study are expected to promote the development of alternative testing methods, as well as achieve regulatory acceptance and implementation of in vitro assays.

Abstract

The present study aims at identifying, among six mammalian and fish cell lines, a sensitive cell line whose azoxystrobin in vitro median inhibitory concentration (IC$_{50}$) better matches the azoxystrobin in vivo short-term Sparus aurata (Gilthead seabream) median lethal concentration (LC$_{50}$). The sulforhodamine B (SRB) colorimetric assay was used as a measure of cell proliferation after 24, 48 and 72 hours of azoxystrobin incubation. The LC$_{50}$/IC$_{50}$ ratio was determined for all cell lines in order to find which cell line better matches fish lethality test results. Several endpoints were then tested using the selected cell line: (1) metabolic function and cellular fitness by the resazurin reduction assay, (2) mitochondrial membrane electric potential (ΔΨm) using the mitochondrial marker tetramethylrhodamine ethyl ester perchlorate (TMRE), and (3) mitochondrial superoxide anion radical production by mitochondria using the MitoSOX fluorescent assay. In parallel, fluorescence microscopy was performed to image ΔΨm and superoxide anion production in mitochondria after 48 and 72 hours of azoxystrobin exposure. Statistical results
were relevant for most cell lines after 48 hours of azoxystrobin exposure after cell proliferation were measured, being H9c2 the most sensitive cells, as well as the ones which provided the best prediction of fish toxicity, with a LC\textsubscript{50,96h}/IC\textsubscript{50,4h} = 0.581. H9c2 cell proliferation upon 72 hours of azoxystrobin exposure revealed a LC\textsubscript{50,96h}/IC\textsubscript{50,72h} = 0.998. Therefore, identical absolute sensitivities were attained for both \textit{in vitro} and \textit{in vivo} assays. To conclude, the H9c2 cell-based assay is reliable and represents a suitable ethical alternative to conventional fish assays for azoxystrobin, and could be used to get valuable insights into the toxic effects of other pesticides.

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**Materials and methods**

Analytical standards and solutions

For the fish lethal test, an azoxystrobin stock solution (1.0 g L\textsuperscript{-1}) was prepared in acetone p.a. (Sigma-Aldrich 32201) and stored at -18\textdegree C. Exposure solutions were prepared in filtered (11 µm, Whatman 1001-047) natural seawater on the day of use according to the ASTM E729 (2002) guideline. No analytical measurements were performed to the exposure solutions. All concentrations are, therefore, presented as nominal values. Solvent control was also prepared on the day of use with acetone (1.14 ml L\textsuperscript{-1}) in filtered natural seawater. For cell-based assays, azoxystrobin stock solutions were prepared in DMSO (Sigma-Aldrich D2650) and stored at 4\textdegree C. Exposure solutions were prepared on the day of use in filtered (0.2 µm sterilized cellulose nitrate filter, Sartorius 11407-47-ACN) Dulbecco's modified Eagle's medium (DMEM)-high glucose (Sigma-Aldrich D5648) adjusted to contain 1.8 g L\textsuperscript{-1} of sodium bicarbonate and supplemented with 10% foetal bovine serum (FBS, Gibco 10270-106) and 1% antibioticantimycotic (Gibco 15240-062), at pH 7.3.
Solvent control medium was prepared on the day of use with DMSO in DMEM-high glucose prepared as previously described.

Fish lethal test

Fish median lethal concentration (LC$_{50,96h}$) was determined according to ASTM E729 (2002) guideline using juveniles (≈5.0 g) of gilthead seabream S. aurata from a commercial fish farm (MARESA, Spain). Fish were maintained in a temperature controlled room (19 ± 1°C) under a natural light regime, and in aerated recirculating aquatic systems composed of round 200-L tanks supplied with natural seawater at 18°C and an appropriate life support system. All fish were from the same batch and were kept under laboratory acclimation conditions for 20 days with an initial density of 3.6 kg m$^{-3}$. Physico-chemical acclimation conditions such as salinity, pH and dissolved oxygen were measured daily in the water using WTW probes. Fish were fed daily with Aqualgold 3 (Sorgal), and were starved for the 24 hours prior to the test to ensure that all animals were at a similar starting point. After the acclimation period, seven active and externally undamaged fish were selected for each of the six azoxystrobin exposure treatments performed in a geometric series with a factor of 1.5, as well as for the negative (fish in filtered natural seawater alone) and solvent controls. The selected fish, weighing from 3.6 to 10.7 g ww, were placed individually into 3-L glass flasks (15 cm Ø). Each flask was aerated using PTFE tubing (Bola S1810-18) and air filtration was carried out by using a syringe filter (0.2 µm, Sartorius 17761-Q). The test room was maintained at the same environmental conditions as the acclimation room. Since azoxystrobin is considered stable to hydrolysis (US-EPA, 1997), a 96-h static non renewal test was performed. Physico-chemical experimental conditions such as salinity, pH and dissolved oxygen were measured at 0, 48 and 96 hours using WTW probes. Fish behaviour and mortality were monitored daily. At the end of the test, total ammonia nitrogen (N-NH$_4$) was
determined in water samples following the Limnologisk metodik (1992) methodology. The amount of toxic (un-ionized) ammonia nitrogen (N-NH₃), which is a function of temperature (18ºC), pH (mean value of 7.7) and salinity (34), was determined according to Spotte and Adams (1983). At the end of the test, surviving fish were over-anesthetized with tricaine methane sulfonate (MS-222, Pharmaq Vm 11003/4013) using a solution of 200 mg L⁻¹ (buffered at pH 7.0-7.5 with sodium bicarbonate) (AVMA/AV-303, 2013).

Cell culture

Mammalian cell lines A549 (human alveolar basal epithelial carcinoma cells), HepG2 (human hepatocellular carcinoma cells), BJ (human skin fibroblast cells) and H9c2 (rat cardiomyoblast cells) were purchased from European Collection of Authenticated Cell Cultures (ECACC) and American Type Culture Collection (ATCC). Fish cell lines VSa16 (vertebra-derived cells) and ABSa15 (branchial arch-derived cells), both derived from S. aurata, were developed in the laboratory of M.L. Cancela by Pombinho et al. (2004) and Marques et al. (2007). All cell lines grew as adherent monolayer and were subcloned at confluence using a phosphate-buffered saline (PBS) solution to wash the cells and 0.05% trypsin-EDTA (Gilco 25300-062) to detach them from the plate. Mammalian and fish cell lines were incubated in a humidified atmosphere with 5% of CO₂ at 37ºC, and with 10% of CO₂ at 33ºC, respectively.

Both VSa16 and ABSa15 fish cell lines had never been used in toxicological studies. Thereby, growth curves of both cell lines were generated to determine some of its characteristics (e.g., doubling time, growth rates). For this purpose, VSa16 and ABSa15 were grown to confluence in T-75 flasks (VWR 734-2313), trypsinised, counted using an automated cell counter (TC₂₀, Bio-Rad), and seeded (VSa16: 10⁴ cells ml⁻¹ density at passages #72, #74 and #75; ABSa15: 5×10³ cells
ml\(^1\) density at passage #75) in 48-well plates (VWR 734-2326). The cell culture medium was changed every other day. Six independent experimental replicates were prepared (N = 6) for each cell line. In order to obtain quantitative cell proliferation data, the SRB colorimetric assay was performed after 16 days of cell culture. This assay was established by Skehan et al. (1990) and further optimised by Papazisis et al. (1997) and Vichai and Kirtikara (2006). SRB is a bright pink aminoxanthene dye which stains cellular proteins, being the amount of dye bonded to cellular proteins proportional to cell number (Vichai and Kirtikara, 2006). The detailed procedure is described below since the SRB assay was also used here to test the cell growth inhibition potential of azoxystrobin. Cell doubling time and growth rates of both fish cell lines were determined from SRB absorbance data using the Doubling Time software version 1.0.10 (http://www.doubling-time.com). At the mentioned experimental conditions, the analysis of growth curves showed a similar dynamics in the development of both cell cultures, since doubling times of 4.0 and 3.6 days were found for VSa16 and ABSa15, respectively. Also, the growth rates were 0.17 and 0.19 for VSa16 and ABSa15, respectively. Therefore, in order to achieve a Log-phase within 24/48 hours to test the cell growth inhibition potential of azoxystrobin, the initial density was increased for both cell lines.

Sulforhodamine B assay

The cell growth inhibition potential of azoxystrobin was evaluated by the SRB assay. Cell lines were seeded (500 µL) in 48-well plates (VWR 734-2326) following the conditions established in Table 26. Then, 24 hours after seeding, cells were treated with azoxystrobin (see concentration range in Table 26). For each independent experiment, negative (cells with media alone) and solvent controls were considered. After exposure to azoxystrobin, cells were washed with PBS and dried, and then fixed for 60 min. at 18ºC with cold 1% acetic acid prepared in methanol. Fixative
was removed and cells were stained for 45 min. at 37ºC using SRB solution (prepared in 1% of acetic acid) and excess of dye was removed by washing the wells at least four times with 1% of acetic acid. Protein-bound dye was dissolved under gentle stirring using 10 mM Tris/base (Sigma-Aldrich T1503) and quantified from absorbance measurements (545 nm) using a microplate reader (Vitor X3, PerkinElmer).

Resazurin reduction assay

The resazurin reduction assay was performed to evaluate the toxic effects of azoxystrobin using both the metabolic function and cellular health as an endpoint. Viable cells with active metabolism reduce resazurin into the pink and fluorescent resorufin product whose fluorescence output is proportional to the changes of cellular redox activity (O’Brien et al., 2000). The working solution was prepared in DMEM-high glucose immediately prior to use and contained 10 mg ml⁻¹ of resazurin (Sigma-Aldrich R7017, stock solution was prepared at 1 mg ml⁻¹ in PBS). Resazurin was added to H9c2 cells (10⁴ cells ml⁻¹ density, passage #16) previously incubated for 48 hours to different concentrations of azoxystrobin (200-12,800 mg L⁻¹). This procedure was performed in 96-well plates (VWR 734-2327), which were incubated for 3.5 hours at 37ºC. Five independent experimental replicates (N = 5) were prepared in the same plate, and for each, negative and solvent controls were

<table>
<thead>
<tr>
<th>Cell line</th>
<th>N*</th>
<th>Passages used</th>
<th>Cells ml⁻¹ density</th>
<th>Concentration range AZX (g L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammalian</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A549 (24 and 48 h assays)</td>
<td>6</td>
<td>12/13</td>
<td>3×10⁴</td>
<td>100-12,800</td>
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<td>HepG2 (24 and 48 h assays)</td>
<td>6</td>
<td>23</td>
<td>3×10⁴</td>
<td>100-51,200</td>
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<tr>
<td>BJ (24 and 48 h assays)</td>
<td>6</td>
<td>10</td>
<td>2×10⁵</td>
<td>800-51,200</td>
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<tr>
<td>H9c2 (24 and 48 h assays)</td>
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<td>13-20</td>
<td>10⁶</td>
<td>100-12,800</td>
</tr>
<tr>
<td>H9c2 (72 h assay)</td>
<td>2</td>
<td>23</td>
<td>10⁶</td>
<td>100-51,200</td>
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<tr>
<td>Fish</td>
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<td>VSa16 (24 and 48 h assays)</td>
<td>4</td>
<td>74/75</td>
<td>4×10⁴</td>
<td>100-51,200</td>
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<td>ABSa15 (24 and 48 h assays)</td>
<td>6</td>
<td>73</td>
<td>3×10⁵</td>
<td>100-51,200</td>
</tr>
</tbody>
</table>

*No. of independent experimental replicates

Resazurin reduction assay

The resazurin reduction assay was performed to evaluate the toxic effects of azoxystrobin using both the metabolic function and cellular health as an endpoint. Viable cells with active metabolism reduce resazurin into the pink and fluorescent resorufin product whose fluorescence output is proportional to the changes of cellular redox activity (O’Brien et al., 2000). The working solution was prepared in DMEM-high glucose immediately prior to use and contained 10 mg ml⁻¹ of resazurin (Sigma-Aldrich R7017, stock solution was prepared at 1 mg ml⁻¹ in PBS). Resazurin was added to H9c2 cells (10⁴ cells ml⁻¹ density, passage #16) previously incubated for 48 hours to different concentrations of azoxystrobin (200-12,800 mg L⁻¹). This procedure was performed in 96-well plates (VWR 734-2327), which were incubated for 3.5 hours at 37ºC. Five independent experimental replicates (N = 5) were prepared in the same plate, and for each, negative and solvent controls were
considered in duplicate. Top well fluorescence was monitored at 37°C, at 540 nm excitation wavelength and 590 nm emission wavelength, and photomultiplier tube (PMT) gain at 70 in a Cytation 3 multiplate reader (Biotek Instruments).

**TMRE assay**

In the present study, ΔΨ<sub>m</sub> was determined by staining H9c2 cells with TMRE followed by fluorescence measurements in a plate reader. Cell handling procedures were similar to the ones used in the resazurin reduction assay, except that cells were seeded (100 µL) in a black/clear flat bottom 96-well plate (BD Falcon 353219) at passage #10. Six independent experimental replicates (N = 6) were prepared in the same plate, and for each, negative and solvent controls were considered in duplicate. Cells were exposed for 48 hours to azoxystrobin, washed with a buffer solution (120 mM NaCl, 5 mM NaHCO<sub>3</sub>, 1.2 mM Na<sub>2</sub>SO<sub>4</sub>, 3.5 mM KCl, 0.4 mM KH<sub>2</sub>PO<sub>4</sub>, 25 mM C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>, 20 mM Hepes, at pH 7.4), and incubated for 45 min. with 50 nM TMRE (Santa Cruz sc-213026, stock solution prepared as 50 µM in DMSO) prepared in buffer solution. In order to validate mitochondrial depolarisation by using TMRE, a positive control was considered for each replicate, using a final concentration of 20 µM of p-trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP, Santa Cruz sc-203578, stock solution prepared as 1 mM in DMSO) 10 min. prior to the TMRE treatment. Fluorescence was monitored at 37°C, at 549 nm excitation wavelength and 575 nm emission wavelength, PMT gain at 70, and measurements made from the bottom of the plate in a Cytation 3 multiplate reader (Biotek Instruments).

**MitoSOX fluorescent assay**

The effect of azoxystrobin on the production of mitochondrial superoxide anion by H9c2 cells was measured by using the fluorescence probe MitoSOX Red (Life
Technologies M36008). Once in the mitochondria, the MitoSOX Red reagent is readily and specifically oxidised by superoxide and the oxidation product becomes highly fluorescent upon binding to nucleic acids. Cell handling procedures were similar to the ones used in the TMRE assay. Cells were exposed for 48 hours to azoxystrobin, washed with buffer solution and then incubated for 30 min. with 5 µM MitoSox Red (stock solution prepared as 5 mM in DMSO) in buffer solution. Cells were washed again, and then dried. Fluorescence was monitored at 37ºC, at 510 nm excitation wavelength and 580 nm emission wavelength, PMT gain at 70, and measurements made from the bottom of the plate in a Cytation 3 multiplate reader (Biotek Instruments).

Fluorescent microscopy

To visually evaluate the effect of azoxystrobin on ΔΨm and superoxide anion formation, H9c2 cells at passage #10 were seeded at a density of $10^4$ cells ml$^{-1}$ in 6-well plates (Thermo Scientific 130184) with a glass coverslip (Menzel BB018018A1) in each well. Two independent experimental replicates (N = 2) were performed and solvent controls were considered for each. At 24 hours post-seeding, cells were treated with either azoxystrobin fish LC$_{50}$ (729 µg L$^{-1}$) or the maximum azoxystrobin concentration tested (51,200 µg L$^{-1}$). Changes in mitochondrial status were observed at 48 and 72 hours after azoxystrobin exposure using TMRE (50 nM) and MitoSox (5 µM) probes, as well as Hoechst 33342 (1 µg) (Invitrogen, Carlsbad, CA) to label nucleus. Imaging was carried out on a Nikon eclipse TieS fluorescence microscope (2012) for TMRE and a Carl Zeiss LSM710 confocal microscope for MitoSox.
Statistical analysis and validity criteria

Gilthead seabream LC \(_{50,96h}\) and 95% confidence intervals were determined from the 96-h mortality records using the six azoxystrobin nominal concentrations (150-1,139 µg L\(^{-1}\)) by probit analysis (on-line Probit 1.63 software).

One-way ANOVA was used to test the significance of the differences between negative and solvent controls in all the cell-based assays performed, as well as between negative and positive controls in the TMRE assay. For the latter assay, ANOVA was followed by a post-hoc Dunnett's test. One-way ANOVA was also used to test differences between negative controls and the maximum stimulatory effects observed at low concentrations of azoxystrobin on HepG2, H9c2 and ABSa15 cells (Zar, 2010). ANOVA tests were performed after the verification of normality and homogeneity assumptions. Whenever the mentioned assumptions were not met, the nonparametric equivalent Kruskal-Wallis test was used. A value equal or inferior to 0.05 was considered statistically significant. Both tests were performed using STATISTICA 7.0 software.

To determine the IC\(_{50s}\) and 95% confidence intervals, the results of the cell-based assays (absorbance or fluorescence data) were expressed as a fraction of the controls. Then, a four-parameter logistic regression after log-transformation of x-axis values (azoxystrobin concentrations) was applied. These analyses were carried out using GraphPad Prism 6.0 software. The strength of the coefficient of determination (\(r^2\) values) was used to determine which of the 24- or 48-h relationships best fits the regression model used.

Individual data points of concentration-response cytotoxicity charts are presented as the arithmetic mean ± standard deviation (SD) using absorbance data at 545 nm. In cellular assays, CV (%) were calculated for the negative controls to ascertain reproducibility and as plate acceptance criteria. CV was calculated by the equation:
CV = (SD/√N)/arithmetic mean × 100

where SD is the standard deviation and N the number of negative control wells per independent experiment. The acceptance criterion is that the CV of each test must be less than or equal to 20%.

**Results**

During the fish lethality test no mortality was observed in negative or solvent controls. The un-ionized ammonia nitrogen present in the samples at the end of the test ranged between 0.004 and 0.028 mg L⁻¹ N-NH₃, which was below the value usually accepted for safe conditions of marine species (0.05 mg L⁻¹ N-NH₃). Gilthead seabream probit LC₅₀ (95% confidence interval) is 729 (585-944) µg L⁻¹ azoxystrobin.

The effect of azoxystrobin on the six cell lines (A549, HepG2, BJ, H9c2, VSa16 and ABSa15) was firstly studied using the SRB assay as a time- and dose-response experiment after 24 and 48 hours, at concentrations between 100 and 51,200 µg L⁻¹. Results showed that all the assays were accepted (validated) since the CV of negative controls never exceeded 13% (Table 27). Moreover, except for BJ cells at 24 hours, all responses showed no significant differences between negative and solvent control treatments, with P_{24h} ≥ 0.089 and P_{48h} ≥ 0.060.

### Table 27: Quality control of sulforhodamine B assays. The coefficients of variation of the mean (CV, in percentage) for the negative controls and the statistically significant differences between negative (C) and solvent (SC) controls are presented. Bold indicates significance.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CV max (%)</td>
<td>C vs SC</td>
<td>CV max (%)</td>
</tr>
<tr>
<td><strong>Mammalian</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A549</td>
<td>13</td>
<td>R(1)=3.312, p=0.089</td>
<td>13</td>
</tr>
<tr>
<td>HepG2</td>
<td>10</td>
<td>R(1)=0.404, p=0.529</td>
<td>5</td>
</tr>
<tr>
<td>BJ</td>
<td>7</td>
<td>R(1)=10.673, p=0.002</td>
<td>4</td>
</tr>
<tr>
<td>H9c2</td>
<td>9</td>
<td>R(1)=0.815, p=0.380</td>
<td>8</td>
</tr>
<tr>
<td><strong>Fish</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VSa16</td>
<td>4</td>
<td>R(1)=0.186, p=0.670</td>
<td>8</td>
</tr>
<tr>
<td>ABSa15</td>
<td>9</td>
<td>R(1)=0.196, p=0.660</td>
<td>13</td>
</tr>
</tbody>
</table>
Except for A549 cells, which presented a low $r^2$ at 48 hours and did not fit the regression model used at 24 hours, and VSa16 cells, which presented low $r^2$ both at 24 and 48 hours, a time-dependent effect of azoxystrobin was observed in all other cell lines (Table 28). Hence, from now on, only HepG2, BJ, H9c2 and ABSa15 cell results were considered for further analysis. Only for these cell lines did statistical analysis reveal relevant results after 48 hours of cells exposure, with $r^2 = 0.908-0.961$. The exception is HepG2, which already presented good results at 24 hours ($r^2 = 0.924$). The 48-h cytotoxicity results were also dose-dependent and data suggests that, except for the BJ cell line, there is a significant ($P \leq 0.004$) stimulatory effect of azoxystrobin at the lowest concentrations (Fig. 20). Depending on the cell line, the magnitude of the maximum stimulatory response was 27-119% greater than controls (mean of negative and solvent controls). IC$_{50,48h}$ (95% confidence interval) results obtained for all the cell lines used are presented in Table 28, with H9c2 cells being the most sensitive and BJ cells the least sensitive to azoxystrobin.

**Table 28** Azoxystrobin time- and dose-response results presented as IC$_{50}$ data after the sulforhodamine B assay.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$ (95% CI)</td>
<td>$r^2$</td>
<td>IC$_{50}$ (95% CI)</td>
</tr>
<tr>
<td>Mammalian</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A549</td>
<td>*</td>
<td>*</td>
<td>6811 (2269-20,445)</td>
</tr>
<tr>
<td>HepG2</td>
<td>1853 (1277-2691)</td>
<td>0.924</td>
<td>1328 (1137-1550)</td>
</tr>
<tr>
<td>BJ</td>
<td>4123 (1508-11,274)</td>
<td>0.304</td>
<td>8245 (6880-9880)</td>
</tr>
<tr>
<td>H9c2</td>
<td>338 (63-1811)</td>
<td>0.540</td>
<td>1255 (1020-1544)</td>
</tr>
<tr>
<td>Fish</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VSa16</td>
<td>362 (157-839)</td>
<td>0.627</td>
<td>3532 (2189-5699)</td>
</tr>
<tr>
<td>ABSa15</td>
<td>2721 (1990-3720)</td>
<td>0.771</td>
<td>2543 (2162-2990)</td>
</tr>
</tbody>
</table>

CI, confidence interval
* data did not fit the model use
Dose-dependent effect on H9c2, ABSa15, BJ and HepG2 cell lines after 48 hours of azoxystrobin exposure and using the SRB assay to assess cell proliferation results. Data presented are the mean ± SD of six replicates.

Taking into account the cell proliferation endpoint studied, the outcomes of LC\(_{50,96h}\)/IC\(_{50,48h}\) revealed that H9c2 is the cell line that better matched the results of the gilthead seabream lethality test (Table 29). Also, considering the azoxystrobin LC\(_{50}\) values reported in literature for the different species of fish presented in the introduction section, the same cell line would still be selected (Table 29).

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Sparus aurata</th>
<th>Cypriodon variegatus</th>
<th>Oncorhynchus mykiss</th>
<th>Lapomis macrochirus</th>
<th>Clupeonella salmonea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammalian</td>
<td>HepG2</td>
<td>0.549</td>
<td>0.505</td>
<td>0.354</td>
<td>0.828</td>
</tr>
<tr>
<td></td>
<td>BJ</td>
<td>0.088</td>
<td>0.081</td>
<td>0.057</td>
<td>0.133</td>
</tr>
<tr>
<td></td>
<td>H9c2</td>
<td>0.581</td>
<td>0.534</td>
<td>0.375</td>
<td>0.877</td>
</tr>
<tr>
<td>Fish</td>
<td>ABSa15</td>
<td>0.287</td>
<td>0.264</td>
<td>0.185</td>
<td>0.433</td>
</tr>
</tbody>
</table>

Therefore, the H9c2 cell line after a 48-h azoxystrobin exposure was used to determine cell viability, mitochondrial membrane polarisation, and superoxide anion production endpoints. The CV of the negative controls never exceeded 15%, thus validating the abovementioned tests. Moreover, mitochondrial depolarisation by the positive control (FCCP) used in the TMRE assay significantly increased mitochondrial TMRE fluorescence (Dunnett's test, \(P < 0.0001\)). Hence, the TMRE concentration used was above the quenching level. The statistical analysis of all
these tests showed no significant differences between negative and solvent control treatments, with $P \geq 0.092$. The cytotoxicity of azoxystrobin in all the experiments performed was very similar, with IC$_{50}$ values within a factor less than 1.5 (Table 30). Therefore, proliferation data were assessed after 72 hours of azoxystrobin incubation by the SRB assay. Quality control and the IC$_{50}$ value of this assay are presented in Tables 27 and 28, respectively. The ratio LC$_{50,96h}$/IC$_{50,72h}$ for azoxystrobin was 0.998.

**Table 30** H9c2 IC$_{50}$ results after 48-h incubation with azoxystrobin.

<table>
<thead>
<tr>
<th>Assay</th>
<th>IC$_{50}$ (95% CI)</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulforhodamine B</td>
<td>1255 (1020-1544)</td>
<td>0.908</td>
</tr>
<tr>
<td>Resazurin</td>
<td>1582 (1407-1779)</td>
<td>0.961</td>
</tr>
<tr>
<td>TMRE</td>
<td>1302 (948-1789)</td>
<td>0.877</td>
</tr>
<tr>
<td>MitoSOX</td>
<td>1515 (1241-1851)</td>
<td>0.913</td>
</tr>
</tbody>
</table>

CI, confidence interval

Fluorescent labelling techniques (TMRE and MitoSox) in combination with live cell imaging revealed that the results of azoxystrobin effects were similar after 48 and 72 hours of exposure on H9c2 cardiomyoblasts. Moreover, TMRE labelled-mitochondria showed a well-defined filamentous network morphology (Fig. 21 A), with MitoSox not detecting superoxide anion production (Fig. 21 B) on the solvent controls. Furthermore, only after exposure to the highest concentration of azoxystrobin did mitochondrial fragmentation and depolarisation occur (TMRE, Fig. 21 E). However, the azoxystrobin LC$_{50}$ concentration tested was capable of increasing superoxide anion production, as well as the highest concentration tested (MitoSox, Fig. 21 D and F).
Fig. 21 Fluorescent microscopy images showing the polarised mitochondrial network (left) and the production of mitochondrial superoxide anions (right) in H9c2 cells after 72 hours of azoxystrobin exposure. A and B: solvent controls (SC), C and D: cells treated with 729 µg L$^{-1}$ of azoxystrobin, and E and F: cells treated with 51,200 µg L$^{-1}$ of azoxystrobin.

**Discussion**

The present study compared the response of *in vitro* cell-based assays using four mammalian and two fish cell lines with the juvenile gilthead seabream *in vivo* lethal test in order to contribute to the development of testing methods alternative to the use of laboratory animals. In general, our results revealed a time-dependent cytotoxicity effect of azoxystrobin. However, as cell lines were developed to mimic different specific stages/situations of interest, cellular specificities were found, possibly due to the different times needed for effective azoxystrobin cell internalisation. Furthermore, azoxystrobin revealed the capacity to inhibit cell proliferation in a concentration dependent manner in HepG2 cells within 24 hours, while most of the cell lines showed the same result only after 48 hours. A549 and VSa16 cell lines are likely to need more than 48 hours for azoxystrobin to pass through the cellular membrane as they both showed low $r^2$, with A549 not even
fitting the regression model used with 24-h data. On the other hand, most carcinoma cells develop mechanisms of multidrug resistance, based on the existence of transporter proteins (e.g., MDR1, MRP1/2, BCRP/MXR), and the human lung adenocarcinoma epithelial cell line A549 is considered a multidrug resistance cell line expressing MRP1 and BCRP proteins (Lebedeva et al., 2011). The comparison of the six IC\(_{50}\) results revealed that the H9c2 cell line is more sensitive to azoxystrobin than the others, since it showed cytotoxicity at smaller concentrations. Conversely, BJ was the least sensitive cell line studied. Therefore, and taking into account all the existing azoxystrobin fish LC\(_{50}\) values, the H9c2 cell line was considered the most appropriate model for in vitro azoxystrobin toxicity testing. Having found a sensitive cell model is of great importance since the risk of false negatives, previously reported as a limitation for the use of cell based assays as an alternative to fish tests (Castaño et al., 2003; Schirmer, 2006), may decrease. Promising results were also reported by Abdul Majeed et al. (2013, 2014) after exposing roho labeo (Labeo rohita) gill cells to malathion (organophosphate insecticide) and after exposing haruan (Channa striatus) gill cells to endosulfan (organochlorine insecticide). LC\(_{50,96h}/IC_{50,24h}\) ratios of 1.001 and 0.984 were found in these studies, both using the Neutral Red assay for malathion and endosulfan, respectively. Taju et al. (2012) also reported a LC\(_{50,96h}/IC_{50,48h}\) ratio of 1.398 after exposing pearlspot cichlid (Etroplus suratensis) eye cells to a tannery effluent. Cytotoxicity in this study was assessed by the Coomassie Blue protein assay.

The similar IC\(_{50,48h}\) results obtained in the present study after multiple endpoints (cell proliferation, cell viability, mitochondrial membrane polarisation and superoxide anion production) were not unexpected, since azoxystrobin interrupts the electron flow at the quinol-oxidizing center (Qo) of complex III (bc1 complex) of the mitochondrial respiratory chain (EFSA, 2010). The same result was also found in Majeed and co-authors’ studies, with IC\(_{50,24h}\) results varying within a factor of 1.1 and
1.2 for malathion and endosulfan, respectively (Abdul Majeed et al., 2013, 2014). Also, when measuring different endpoints in three cell lines derived from eye, kidney and gill tissue of pearlspot cichlid for the toxicity evaluation of a tannery effluent, results revealed IC\textsubscript{50,48h} variations within a factor of 3.3 for the eye cell line, and within a factor of 1.3 for both the kidney and gill cell lines (Taju et al., 2012).

The relatively common biological phenomenon characterized by low dose stimulation and high dose inhibition was observed in HepG2, H9c2 and ABSa15 cells. This apparent hormetic effect is possible due to the fact that azoxystrobin was inspired by the structure and activity of a naturally occurring compound, the fungicide strobilurin A (Sauter et al., 1999). In nature, organisms are known to have some plasticity to cope with low levels of chemical stress by activating the repair mechanisms of the body, thus producing an overcompensation response. The hormesis concept applied to routine testing of hazards was comprehensively studied by Bailer and Oris (1998). Similarly, several other studies reported the same effect regarding the cytotoxicity of pharmaceuticals (Caminada et al., 2006), metals (Shúilleabhain et al., 2004) and nanoparticles (Jiao et al., 2014).

The present study highlighted the potential of azoxystrobin to decrease H9c2 cell viability, possibly due to the observed collapse of mitochondrial transmembrane potential, which may result from the inhibition of the mitochondrial respiratory chain caused by azoxystrobin. This blockage may also cause an increased production of ROS by the mitochondrial respiratory chain, which was corroborated by vital epifluorescent microscopy images (Fig. 21), as well as by MitoSOX assay results, thus showing azoxystrobin concentration-dependently increased mitochondrial superoxide anion generation in H9c2 cells. This production of ROS at the mitochondrial complex III is similar to what occurs with the antifungal antibiotic myxothiazol, as was demonstrated by Starkov and Fiskum (2001). In parallel, mitochondria displayed a fragmented structure after treatment with 51,200 µg L\textsuperscript{-1} of
Azoxystrobin. Mitochondrial fusion is critical for the maintenance of mitochondrial function, as the inhibition of mitochondrial fusion results in a loss of ΔΨm (Chen et al., 2003). Azoxystrobin-triggered oxidative stress can also explain mitochondrial fragmentation, as Wu et al. (2011) observed after studying cellular alterations of mitochondrial oxidative stress caused by high-fluence low-power laser irradiation.
Chapter V – Azoxystrobin effects at the community level

Rodrigues ET, MA Pardal, C Gante, J Loureiro, I Lopes (under review)


4.529 IF2014; Q1(2014) Pollution
DETERMINATION AND VALIDATION OF AN AQUATIC PREDICTED NO-EFFECT-CONCENTRATION (PNEC) FOR AZOXYSTROBIN

The quantification of the likelihood to occur and severity of adverse effects resulting from the use of pesticides may indicate the nature of the measures which are necessary to reduce environmental risks to an acceptable level. Environmental risks are typically characterised in the risk assessment framework by considering the ratio between exposure concentrations and critical effect concentrations. This approach is currently the most favoured and, in OECD countries, critical effect concentrations are based on PNEC values. The main goal of the following study was to determine and validate an aquatic PNEC value for azoxystrobin. To attain this goal, three specific objectives were delineated:

(1) Determining if the commercial formulation Ortiva® is more toxic than its active ingredient azoxystrobin. To reach this, median effective concentrations (EC$_{50}$ and LC$_{50}$) were determined for species representative of several functional and trophic levels of marine ecosystems.

(2) Comparing the sensitivity of marine species to azoxystrobin with those of freshwater species. A general strategy to assess the risk of pesticides for marine environments consists of applying safety factors to the risk level calculated based on freshwater toxicity data. Since the available ecotoxicological data on azoxystrobin derive mostly from assays with aquatic freshwater species (this project, Rodrigues et al., 2013c), it was possible to generate a SSD curve also for freshwater species and compare both marine and freshwater curves by means of the SSD concept in order to compare sensitivities (Leung et al., 2001).
(3) Determining if PNEC values generated using SSD curves are more protective and conservative than those derived using the AF method. To reach this, SSD and AF approaches were applied to freshwater and marine toxicity datasets for azoxystrobin, as well as to a marine toxicity dataset for Ortiva.

Abstract

Environmental risks are typically characterised in the risk assessment framework by considering the ratio between exposure concentrations and critical effect concentrations. This approach is currently the most favoured and, in the Organisation for Economic Co-operation and Development (OECD) countries, critical effect concentrations are based on PNEC values. The present project determined and validated an aquatic PNEC value for azoxystrobin. Assessment factor and SSD approaches were applied to freshwater and marine toxicity datasets for azoxystrobin, as well as to a marine toxicity dataset for Ortiva®, a commercial formulation of azoxystrobin. After comparing the six PNEC values estimated in the present study to all the laboratory-derived toxicity information available for azoxystrobin, PNEC values derived using the assessment factor method were considered overprotective and a PNEC of 1.0 µg L\(^{-1}\) was recommended for azoxystrobin in the aquatic environmental compartment. This value derived from marine Ortiva toxicity data, which highlights the importance of testing commercial formulations of pesticides to attain realistic toxic effects.

***
Materials and methods

Experimental design for toxicity assays

Median effective concentrations were determined by conducting short-term toxicity assays using both the azoxystrobin analytical standard and the commercial formulation Ortiva®. The selected species were non-pathogenic bacteria (Vibrio fischeri), microalgae (Phaeodactylum tricornutum, Thalassiosira weissflogii, Rhodomonas lens, Nannochloropsis gaditana and Isochrysis galbana), rotifers (Brachionus plicatilis), macrocrustaceans (Artemia franciscana), gastropod molluscs (Rissoa parva and Gibbula umbilicalis) and fish (Solea senegalensis). With a single exception, the R. parva assay, all lethal assays were performed using early life stages, larvae or juveniles, as they generally tend to be more sensitive to pollutants than later life stages (Buchwalter et al., 2004; Mohammed, 2013).

Laboratory material

The laboratory material used to perform the bioassays was all glassware, with the exception of the assays with B. plicatilis, whose material was made of PVC. Prior to use, it was thoroughly washed for at least six hours with a basic detergent (2%, Merck Extran MA01 Alkaline) prepared in distilled water. Intermediate and exposure solutions were prepared in new amber glass vials with PTFE liners in the cap (Supelco 27004).

Analytical standards and solutions

Stock standard solutions were prepared in acetone p.a. and stored at -18°C. The fungicide Ortiva® was kindly provided by the tree nursery Almeida Rodrigues Viveiros Agrícolas (Coimbra, Portugal). Ortiva intermediate solutions and both azoxystrobin and Ortiva exposure media were freshly prepared on the day of use in
reconstituted marine water (tropic marin salt) using ultrapure water purified with a Milli-Q Biocel System at salinities presented in Table 31. In the case of the V. fischeri assay, the exposure medium was prepared in the diluent supplied by Microtox (Modern Water), whereas for B. plicatilis and A. franciscana assays, exposure media were prepared using reagent grade chemicals. For the assays whose exposure media were prepared by serial dilutions (bacteria and microalgae assays), the nominal concentrations of the solution used to start the serial dilutions were confirmed using a validated chemical method. Concerning lethal assays, the nominal concentrations of exposure media (samples collected at the end of the assay) were also confirmed. However, no analyses were performed for B. plicatilis, G. umbilicalis and S. senegalensis assays, since no mortality was observed, some nominal concentrations were below the method’s limit of quantification, and there was no sufficient volume at the end of the assay, respectively.

<table>
<thead>
<tr>
<th>Table 31 Bioassays conditions.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species T (ºC) Photoperiod Salinity Exposure conditions</td>
</tr>
<tr>
<td>--------------------------------</td>
</tr>
<tr>
<td>V. fischeri 4 24 h</td>
</tr>
<tr>
<td>P. marinum 20 24 h L</td>
</tr>
<tr>
<td>T. weissflogii 20 24 h L</td>
</tr>
<tr>
<td>R. yers 20 24 h L</td>
</tr>
<tr>
<td>N. gaditana 20 24 h L</td>
</tr>
<tr>
<td>J. galbana 20 24 h L</td>
</tr>
<tr>
<td>B. plicatilis 25 24 h D</td>
</tr>
<tr>
<td>A. franciscana 25 24 h D</td>
</tr>
<tr>
<td>R. parr 15 16/8 h L/D</td>
</tr>
<tr>
<td>G. umbilicalis 15 16/8 h L/D</td>
</tr>
<tr>
<td>S. senegalensis 20 12/12 L/D</td>
</tr>
</tbody>
</table>

*Temperatures; L: light; D: dark

Chemical analytical methodology

Azoxystrobin analyses in water samples were carried out in the laboratory of the Instituto Superior Técnico (University of Lisbon, Portugal) according to the procedure described in Chapter III.
The concentrations used in the data analysis were attained by calculating the geometric mean of nominal and measured concentrations, as recommended by Traas (2001).

Single-species short-term toxicity assays

Since the hydrolysis rate of azoxystrobin in water is considered stable (US-EPA, 1997), static non-renewal tests were performed in the conditions established in Table 31. A negative and a solvent control, the latter containing the highest concentration of the solvent used, were considered in azoxystrobin bioassays (APHA, 1989), whereas for Ortiva bioassays, only negative controls were performed. Physico-chemical conditions such as salinity, pH and dissolved oxygen were measured in the media at the beginning of each assay using the multi-parameter Hach HQ30d.

The sensitivity of *V. fischeri* to both azoxystrobin and Ortiva was assessed using the bioluminescent assay Microtox, which was coupled with the Omni Windows software. The 81.9% Basic Test was used. In the azoxystrobin assay, the solvent control was tested as 50 µL of acetone in 10 mL of the Microtox diluent, and no bacteria luminescence inhibition was observed. To compute median effective concentrations, only the negative control was considered.

Microalgae were chosen from among four phylogenetic groups of marine phytoplankton: Bacillariophyceae (the pennate diatom *P. tricornutum* and the centric diatom *T. weissflogii*), Cryptophyceae (*R. lens*), Eustigmatophyceae (*N. gaditana*) and Haptophyceae (*I. galbana*). Microalgae cultures were provided by the University of Aveiro & CESAM (Portugal), and were initially supplied by AQUALGAE (Spain). Stock culture maintenance and bioassays were both performed according to the ISO 10253 (2006) guideline. Briefly, stock cultures were maintained axenically in 100 mL-Erlenmeyers with growth medium (50 mL) prepared with reconstituted water.
supplemented with 10 mL L\(^{-1}\) of “optimedium” (AQUALGAE). An extra supplement of sodium silicate (45 µg L\(^{-1}\)) was added to the *T. weissflogii* culture medium. Cultures were placed in a 20ºC constant-temperature cabinet (Binder KBW\(_{400}\)) with illumination programmed to continuous wide-spectrum light from cool daylight lumilux lamps (Osram L18W/865). Light intensity at the surface of the culture vessels was about 3,300 lux (Delta OHM HD9221). An orbital shaker (Heidolph rotamax 120) was used to ensure adequate culture homogenization. To start the assays, algae were inoculated from exponential growth-phase stock cultures and placed in glass test tubes of 4.0 mL and 10 mm Ø covered with parafilm using 1.0 mL of exposure medium prepared by serial dilutions. The assays took place in the same conditions used for maintaining the stock cultures, and the test tubes were vortexed and repositioned daily. At the end of the bioassays, and as recommended by Marie et al. (2014), samples were preserved with glutaraldehyde (Fluka 49632) 0.25% (final concentration) and then deep-frozen (-80ºC, Haier DW-86L628) for subsequent counting by flow cytometry. No preservation was performed in *N. gaditana* and *I. galbana* samples. Flow cytometry analyses were, therefore, made with live cells. All the counts were achieved using the True Volumetric Absolute Counting technique (Partec CyFlow Space flow cytometer). The data acquisition FloMax software was optimized by using both the scatter (forward and side scatter) and the auto-fluorescence properties of the cells. To eliminate debris signal, the auto-fluorescence signal of the cells was used to gate the particles in the forward versus side scatter (both in logarithmic scale). In the cytogram, a region was defined around the cloud of cells for each microalgae species, which was kept constant throughout the analyses. Due to the size of *T. weissflogii* cells, counts were performed using a Neubauer chamber.

The bioassays with *B. plicatilis* were conducted according to the Rotoxkit M\(^{TM}\) (MicroBioTests) protocol, using 24 h-conditioned PVC wells with exposure media
prepared with reconstituted marine water (ASPM) according to the ASTM E 1440 (1991) guideline. The assays were conducted in a 25°C constant-temperature cabinet programmed to continuous darkness.

The bioassays with *A. franciscana* were conducted according to the Artoxkit M™ (MicroBioTests) protocol with minor modifications: the multiwell test plates supplied by the kit were replaced by 4.0 mL glass tubes with 1.0 mL of exposure media. The assays were conducted in a 25°C constant-temperature cabinet programmed to continuous darkness.

Gastropods *R. parva* (adults) and *G. umbilicalis* (juveniles, <8 mm maximum shell diameter ([Gaudêncio and Guerra, 1986](#)) were collected in April (*R. parva*) and May (*G. umbilicalis*) of 2015, during low tide, in an intertidal rocky shore of the Portuguese Atlantic coast (40°10'16.5''N, 8°53'33.6''W). The water temperature at the time of collection was 15°C. According to the Marine Macroalgae Assessment Tool (MarMAT), a multi-metric method to classify the ecological quality status of coastal areas based on marine macroalgae, the selected site was considered of good/high quality ([Neto et al., 2012](#)). The stocks of gastropods were maintained in a 15°C constant-temperature cabinet with illumination programmed to 16-h light (≈1,500 lux)/8-h dark periods. The stocks were maintained in aerated glass tanks of 3.5 L (25 × 20 × 9.5 cm) with 2.0 L of reconstituted water for at least 20 days (acclimation period). Air filtration was done through a 0.2 µm syringe filter and the media were replaced in whole every three days. The organisms were supplied *ad libitum* with fresh *Ulva*, although they were starved in the 48 hours prior to the assays. During the starvation period, 150 mg L⁻¹ of sodium hydrogen carbonate (NaHCO₃, Sigma S5761) was added to the reconstituted water since the carbonate is used by gastropods for their skeletons (shells). To start the assay, *R. parva* were observed under a binocular microscope to perceive mobility and those carrying egg masses were discarded. Concerning *G. umbilicalis*, mobility was observed with the
naked eye. Size-calibrated snails, *R. parva*: 2.4-3.8 mm total length (microscope Leica M-80 with a calibrated ocular micrometer) and *G. umbilicalis*: 6.5-8.1 mm maximum diameter (electronic digital caliper VWR 1819-0012), were randomly introduced in each replicate test vessel (*R. parva*: 50 mL-Erlenmeyer and *G. umbilicalis*: 250 mL-Erlenmeyer) containing exposure media (*R. parva*: 40 mL and *G. umbilicalis*: 150 mL) prepared in NaHCO₃-supplemented reconstituted water. During the assays, each test vessel was covered with a watch glass and checked twice a day, and emerged snails were gently submerged. At the end of the assays, the criterion used to determine mortality was failure to respond to gentle physical stimulation observed under a binocular microscope.

Senegal sole pelagic larvae (newly hatched) *S. senegalensis* were kindly provided by the marine fish farm A. Coelho & Castro (Estela, Portugal). On arrival to the laboratory, larvae were immediately placed in a 20°C constant-temperature cabinet for three hours. Then, they were randomly introduced in each replicate test vessel (4.0 mL glass tubes with 1.0 mL of exposure media). The assays took place in the abovementioned constant-temperature cabinet with illumination programmed to 12-h light (850 lux)/12-h dark periods. The 48-exposure period covered the yolk-sac stage, thus making feeding unnecessary during the assay.

Statistical analysis and validity criteria

In microalgae assays, and using cell density data, the coefficients of variation of the mean (CV) were calculated for the negative controls to ascertain reproducibility and to be used as assay acceptance criteria, according to the equation:

$$CV(\%) = \frac{SD}{\sqrt{N}}/\text{arithmetic mean} \times 100$$

where SD is the standard deviation and *N* the number of negative control vessels per experiment. The acceptance criterion is that the CV of each test must be ≤10%.
As a quality assurance and control measure of *B. plicatilis* and *A. franciscana* assays, according to the Rotoxkit and Artoxkit M protocols, a criterion of 90% control survival was considered to validate the tests. The same criterion was attained for gastropods and fish assays, according to the ASTM E 729 (2002) guideline.

The EC$_{50s}$ and 95% confidence intervals (CI) for *V. fischeri* data were determined using the Omni Windows software by graphing the log of the sample concentration versus the percentage of light decrease. The ErC$_{50s}$ (95% CI) for microalgae growth inhibition data were determined using the standard method ISO 10253 (2006) and the STATISTICA 7.0 software. This software was also used to test, through a t-test, the statistical significance of the difference between negative and solvent controls in the microalgae azoxystrobin assays. Using the same software, a Mann-Whitney U test was used to verify the statistical significance of the difference between marine azoxystrobin and Ortiva toxicity datasets, and between marine and freshwater datasets for azoxystrobin, as well as to verify the statistical significance of the difference between marine and freshwater sensitivities to azoxystrobin within a trophic group (microalgae, invertebrates, and fish). Moreover, the statistical significance of the difference between trophic groups of each dataset were verified by a Mann-Whitney U test (two groups) or by a Kruskal-Wallis test (more than two groups). A value equal or inferior to 0.05 was considered statistically significant. The LC$_{50s}$ (95% CI) from mortality records were determined by probit analysis. The SSD curves and HC$_{5s}$ were generated by the E7X 2.1 software (Van Vlaardingen et al., 2004). Associated with hazardous concentrations, 95% and 50% CIs were also derived by setting the lower limit HC$_5$ (LLHC$_5$) and the median HC$_5$, respectively.

Freshwater toxicity data collection

Data on the toxicity of azoxystrobin to freshwater organisms were compiled from two main sources: scientific literature and the ECOTOX (http://cfpub.epa.gov/ecotox/).
database. This database is internationally recognized as one of the most reliable toxicity databases available (Cronin and Schulz, 2003). All gathered data are reported in Table 32. In order to avoid overrepresentation of toxicity data from one particular species, and as recommended by Newman et al. (2000), the geometric mean was determined for the four D. magna LC50,48h available. Hence, a LC50,48h = 160 µg L−1 was further considered in the SSD curve. Therefore, a total of 13 species, including 4 microalgae, 1 macrophyte, 4 invertebrates (copepod, cladocera and amphipods) and 4 fish, were used to generate the SSD curve.

Table 32 Freshwater short-term toxicity data for azoxystrobin.

<table>
<thead>
<tr>
<th>Species</th>
<th>Group</th>
<th>Endpoint (exposure time)</th>
<th>EC50 and LC50 (95% CI) (µg L−1)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabamia fossarum</td>
<td>microalgae, blue-green</td>
<td>growth (120 h)</td>
<td>13,000 (12,000-14,000)</td>
<td>US-EPA, 2015</td>
</tr>
<tr>
<td>Navicula pelliculosa</td>
<td>microalgae, bacilliform</td>
<td>growth (96 h)</td>
<td>49 (43-58)</td>
<td>US-EPA, 2015</td>
</tr>
<tr>
<td>Pseudokirchneriella subcapitata</td>
<td>microalgae, chlorophyta</td>
<td>growth (96 h)</td>
<td>106 (92-121)</td>
<td>US-EPA, 2015</td>
</tr>
<tr>
<td>Chlorella vulgaris</td>
<td>macrophyte</td>
<td>no. of fronds (14 d)</td>
<td>510 (440-600)</td>
<td>Liu et al., 2015</td>
</tr>
<tr>
<td>Lemna gibba</td>
<td>invertebrate, copepod</td>
<td>immobilization (48 h)</td>
<td>3,400 (3,000-3,900)</td>
<td>Smyth et al., 1993</td>
</tr>
<tr>
<td>Macropodella fuscus</td>
<td>invertebrate, cladocera</td>
<td>mortality (48 h)</td>
<td>130</td>
<td>European Commission, 1998</td>
</tr>
<tr>
<td>Daphnia magna, neonates</td>
<td>invertebrate, cladocera</td>
<td>mortality (48 h)</td>
<td>340 (320-360)</td>
<td>Ochsa-Acufa et al., 2009</td>
</tr>
<tr>
<td>D. magna, dune Gammeimosen, neonates</td>
<td></td>
<td></td>
<td>71 (34-126)</td>
<td>Warning et al., 2009</td>
</tr>
<tr>
<td>D. magna, dune Herlev Gadekær, neonates</td>
<td></td>
<td></td>
<td>98 (66-139)</td>
<td>Warning et al., 2009</td>
</tr>
<tr>
<td>D. magna, dune Langedam, neonates</td>
<td></td>
<td></td>
<td>277 (145-427)</td>
<td>Warning et al., 2009</td>
</tr>
<tr>
<td>Gammarus fossarum, adult males</td>
<td>invertebrate, amphipoda</td>
<td>mortality (7 d)</td>
<td>148 (128-169)</td>
<td>Zubrod et al., 2014</td>
</tr>
<tr>
<td>G. pulce, adults</td>
<td>invertebrate, amphipoda</td>
<td>mortality (96 h)</td>
<td>270 (170-450)</td>
<td>Belkova and Lisz, 2008</td>
</tr>
<tr>
<td>Carassius auratus</td>
<td>fish, cyprinidae</td>
<td>mortality (48 h)</td>
<td>540 (419-771)</td>
<td>Liu et al., 2013</td>
</tr>
<tr>
<td>Chironomus pilotus, adults</td>
<td>fish, salmonidae</td>
<td>mortality (96 h)</td>
<td>470 (400-580)</td>
<td>US-EPA, 1997</td>
</tr>
<tr>
<td>Lepomis macrochirius</td>
<td>fish, centrarchidae</td>
<td>1,100 (900-1,700)</td>
<td>US-EPA, 1997</td>
<td></td>
</tr>
</tbody>
</table>

Estimation of PNEC values

PNEC values were calculated by using two approaches in compliance with the European Chemicals Bureau (2003): SSD and AF methods. The PNEC values obtained by the SSD approach derived from each median HC5 value, which was divided by 5. The PNEC values obtained by the AF approach were calculated by dividing the lowest value of each toxicity dataset by an appropriate assessment factor which corresponds for short-term data, to 1,000.
Results

Results of the microalgal toxicity tests showed that all the assays were validated, since the CV of negative controls never exceeded 9%. With the exception of *N. gaditana*, all the microalgal species tested showed no significant differences between negative and solvent control responses, with $P \geq 0.162$. Concerning *N. gaditana*, there were significant differences between negative and solvent control treatments ($P = 0.028$), but the percentage of difference was lower than 10% (8.6%) and was thus considered negligible. Therefore, the ErC$_{50}$ value for *N. gaditana* was considered in the azoxystrobin SSD curve. The diatom *P. tricornutum* presented low sensitivity to azoxystrobin, showing no growth inhibition up to 5.9 mg L$^{-1}$, which is approximately the value of the azoxystrobin maximum solubility in water (6.7 mg L$^{-1}$, European Commission, 1998). Similarly, data from *T. weissflogii* and *R. lens* only allowed ErC$_{20}$ calculations. Therefore, for those species, in order to determine if Ortiva is more toxic than its active ingredient, the ErC$_{20}$ were calculated also for Ortiva. Thus, for *T. weissflogii*, the ErC$_{20}$ (95% CI) were 5.0 (3.9-6.0) mg L$^{-1}$ and 2.6 (1.9-3.4) mg L$^{-1}$ for azoxystrobin and Ortiva, respectively; while for *R. lens*, the ErC$_{20}$ (95% CI) were 4.7 (3.7-5.7) mg L$^{-1}$ and 2.3 (2.2-2.4) mg L$^{-1}$, respectively. Table 33 comprises the ErC$_{50}$ values of the species which allowed data analysis.

Table 33: Marine short-term toxicity data assessed in the present study, as well as data reported in literature, for azoxystrobin and Ortiva.

<table>
<thead>
<tr>
<th>Species</th>
<th>Group</th>
<th>Endpoint (exposure time)</th>
<th>$EC_{50}$ and $LC_{50}$ (95% CI)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. fisheri</td>
<td>bacteria</td>
<td>luminescence inhibition (5 min)</td>
<td>7.0 (5.9-8.2) µg L$^{-1}$</td>
<td>869 (656-1129) µg L$^{-1}$</td>
</tr>
<tr>
<td>P. tricornutum</td>
<td>microalga, bacillariophyceae</td>
<td>growth inhibition (72 h)</td>
<td>&gt;5.9 mg L$^{-1}$</td>
<td>3.0 (2.8-3.2) mg L$^{-1}$</td>
</tr>
<tr>
<td>T. weissflogii</td>
<td>microalga, bacillariophyceae</td>
<td>growth inhibition (72 h)</td>
<td>&gt;5.4 mg L$^{-1}$</td>
<td>4.3 (3.8-5.1) mg L$^{-1}$</td>
</tr>
<tr>
<td>Skeletonema costatum</td>
<td>microalga, cryptophyceae</td>
<td>growth inhibition (72 h)</td>
<td>300 µg L$^{-1}$</td>
<td>-</td>
</tr>
<tr>
<td>N. gaditana</td>
<td>microalga, euglenophyceae</td>
<td>growth inhibition (72 h)</td>
<td>298 (193-463) µg L$^{-1}$</td>
<td>243 (121-364) µg L$^{-1}$</td>
</tr>
<tr>
<td>J. galbana</td>
<td>microalga, haptophyceae</td>
<td>growth inhibition (72 h)</td>
<td>31 (24-38) µg L$^{-1}$</td>
<td>29 (24-33) µg L$^{-1}$</td>
</tr>
<tr>
<td>B. plicatilis</td>
<td>invertebrate, rotifer</td>
<td>mortality (24 h)</td>
<td>&gt;6.8 mg L$^{-1}$</td>
<td>-</td>
</tr>
<tr>
<td>A. franciscana, larvae</td>
<td>invertebrate, artemiasae</td>
<td>mortality (24 h)</td>
<td>345 (244-454) µg L$^{-1}$</td>
<td>1.3 (1.1-1.5) mg L$^{-1}$</td>
</tr>
<tr>
<td>Chlamydomonas giga</td>
<td>invertebrate, bivalvia</td>
<td>mortality (48 h)</td>
<td>1.3 (1.1-1.4) mg L$^{-1}$</td>
<td>-</td>
</tr>
<tr>
<td>R. panca, adults</td>
<td>invertebrate, gastropoda</td>
<td>mortality (96 h)</td>
<td>118 (100-140) µg L$^{-1}$</td>
<td>-</td>
</tr>
<tr>
<td>G. umbilicalis, juveniles</td>
<td>invertebrate, crustacea</td>
<td>mortality (96 h)</td>
<td>13 (10-16) µg L$^{-1}$</td>
<td>17 (13-22) µg L$^{-1}$</td>
</tr>
<tr>
<td>S. zeamais, larvae</td>
<td>fish, ostracidae</td>
<td>mortality (48 h)</td>
<td>698 (576-855) µg L$^{-1}$</td>
<td>1.3 (1.2-1.3) mg L$^{-1}$</td>
</tr>
<tr>
<td>Cyprinodon variegatus</td>
<td>fish, cyprinodontidae</td>
<td>mortality (96 h)</td>
<td>671 (560-800) µg L$^{-1}$</td>
<td>-</td>
</tr>
<tr>
<td>Sparus aurata, juveniles</td>
<td>fish, sparidae</td>
<td>mortality (96 h)</td>
<td>729 (585-944) µg L$^{-1}$</td>
<td>-</td>
</tr>
</tbody>
</table>
Concerning lethal tests, mortality was below the acceptance criterion of 10% in all the assays. The rotifer *B. plicatilis* presented low sensitivity to both azoxystrobin and Ortiva, showing no mortality up to nominal concentrations of 6.8 mg L\(^{-1}\) and 6.2 mg L\(^{-1}\) for azoxystrobin and Ortiva, respectively. Therefore, no LC\(_{50}\)s were determined for this species. For all the other assays, time-dependent LC\(_{50}\) values are presented in Table 33. This table also includes azoxystrobin short-term toxicity data regarding marine species reported in literature. The range of values for the azoxystrobin toxicity dataset varies from 13 to 6,961 µg L\(^{-1}\), whereas the range of values is from 17 to 868,681 µg L\(^{-1}\) for the Ortiva toxicity dataset. In general, among the studied species, toxicity results showed several orders of magnitude for both azoxystrobin and Ortiva datasets (Table 33). Therefore, data analysis on toxicity data revealed no statistical differences between azoxystrobin and Ortiva datasets, being \(P = 0.227\). Also, concerning differences in sensitivity by trophic group in each dataset, the results indicated that there are no differences in azoxystrobin (\(P = 0.174\)) or in Ortiva (\(P = 0.245\)). The gastropod *G. umbilicalis* was the most sensitive species to both azoxystrobin and Ortiva. Conversely, the bacterium *V. fischeri* was the least sensitive species to both azoxystrobin and Ortiva (Table 33).

Concerning the freshwater toxicity data reported in Table 32, the concentration range of values varied between 49 and 13,000 µg L\(^{-1}\). Data analysis showed that different trophic groups (microalgae, invertebrates and fish) did not exhibit different sensitivities to azoxystrobin, with \(P = 0.155\). The planktonic species *Navicula pelliculosa* and *Anabaena flosaquae* were the most and the least sensitive freshwater species to azoxystrobin, respectively.

The graphical representation of the SSD curve for freshwater species is presented in Fig. 22 A. Concerning cumulative frequency distributions for marine species, a total of 12 species (7 from this study and 5 gained from literature), including 1 bacterium, 3 microalgae, 5 invertebrates (from which 2 are crustaceans) and 3 fish,
were used to generate the SSD curve for azoxystrobin (Fig. 22 B), whereas a total of 9 species (all from this study), including 1 bacterium, 5 microalgae, 2 invertebrates (from which 1 is a crustacean) and 1 fish, were used to determine the curve for Ortiva (Fig. 22 C).

Fig. 22 SSD curves derived from short-term toxicity data for azoxystrobin. Captions detail species sensitivity ranking of each dataset (microalgae, invertebrates, fish, aquatic plant, bacterium).
The comparison of sensitivity of marine and freshwater organisms to azoxystrobin showed that, in general, no significant differences were observed \((P = 0.664)\). Also, no significant differences were observed between marine and freshwater specific trophic groups, microalgae \((P = 0.831)\), invertebrates \((P = 0.624)\) and fish \((P = 1.000)\). A lack of parity between both datasets should be highlighted, since a bacterium \((V. fischeri)\) is present in the marine dataset, and an aquatic plant \((L. gibba)\) is present in the freshwater dataset which are do not exist in their counterparts. Nevertheless, representativeness of microalgae, invertebrates and fish is similar. Therefore, in order to visually assess the extent of either congruence or discrepancy between marine and freshwater datasets for azoxystrobin, the two SSD curves were plotted in the same graph (Fig. 23). Results showed a systematic shift of both datasets with similar slopes.

![Comparison of SSD curves for marine and freshwater species.](image)

An overview of freshwater and marine (azoxystrobin and Ortiva) lower limit and median HC₅ values derived from the SSD curves, as well as the PNEC values calculated from both the HC₅ values and using the AF approach, are presented in Table 34.
Table 34 Lower limit and median HC₅ values derived from the SSD curves, as well as PNEC values calculated from both the HC₅ values and using the AF approach.

<table>
<thead>
<tr>
<th></th>
<th>Freshwater AZX (µg L⁻¹)</th>
<th>Marine AZX (µg L⁻¹)</th>
<th>Ortiva (µg L⁻¹ a.i.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower limit HC₅</td>
<td>6.8</td>
<td>2.5</td>
<td>0.077</td>
</tr>
<tr>
<td>Median HC₅</td>
<td>33</td>
<td>15</td>
<td>5.2</td>
</tr>
<tr>
<td>PNEC SSD</td>
<td>6.5</td>
<td>3.0</td>
<td>1.0</td>
</tr>
<tr>
<td>PNEC AF</td>
<td>0.049</td>
<td>0.013</td>
<td>0.017</td>
</tr>
</tbody>
</table>

Discussion

The short-term effects exerted by azoxystrobin and Ortiva were studied in order to determine if Ortiva is more toxic to marine communities than its active ingredient. Concerning the effects of azoxystrobin on both diatoms *P. tricornutum* and *T. weissflogii*, and on the cryptophyceae *R. lens*, as well as on the rotifer *B. plicatilis*, experimentally testing concentrations as high as those required to obtain EC₅₀s or LC₅₀s was impractical due to the low sensitivity of these species. Nevertheless, such high concentrations are of limited ecological relevance since the maximum water concentration of azoxystrobin found in natural environments is 29.7 µg L⁻¹ (Berenzen et al., 2005). In order to compare the toxic effects of azoxystrobin and Ortiva, data evaluation from nine species of four different trophic groups (decomposers, primary producers and consumers and secondary consumers) suggested that azoxystrobin and Ortiva provoke different levels of toxicity to marine species, depending on the species (high interspecific variability in sensitivity). For instance, results showed similar toxicities of azoxystrobin and Ortiva in two microalgae species (*N. gaditana* and *I. galbana*) and in the gastropod *G. umbilicalis*. However, in the cases of bacteria (*V. fischeri*), crustaceans (*A. franciscana*) and fish (*S. senegalensis*), toxicity was primarily due to the active ingredient. Conversely, for the generality of phytoplankton taxa (*P. tricornutum*, *T. weissflogii* and *R. lens*), Ortiva presented higher toxicity than azoxystrobin. Similar sensitivities to azoxystrobin and its commercial formulation Quilt® were also observed for *Bufo cognatus* tadpoles (Hooser et al., 2012). Nevertheless, results of the present study...
suggest that the so-called “inert” ingredients or the declared chemical propane-1,2-diol may have an influence on the toxicity of Ortiva within the microalgae group. Even though no statistical differences were found between marine azoxystrobin and Ortiva datasets, or between trophic groups within a dataset, it was not possible to conclude that marine species respond similarly to azoxystrobin and Ortiva since high interspecific variability in sensitivities was found. Nevertheless, the most sensitive species to both azoxystrobin and Ortiva was the gastropod *G. umbilicalis*, with LC_{50} of 13 and 17 µg L^{-1}, respectively.

Using the SSD key tool to assess the ecotoxicological threat of azoxystrobin and Ortiva to marine biodiversity, the data gathered by the present study concluded that, in order to protect 95% of the species, the water concentration of azoxystrobin cannot exceed 2.5 µg L^{-1}. This value should ensure low risk to marine organisms. However, regarding the Ortiva toxicity dataset, a much more protective concentration value was attained for marine environments (0.077 µg L^{-1}). Therefore, 0.077 µg L^{-1}, as the lowest value obtained, should be the onerecommend as threshold value for azoxystrobin so as to protect marine communities.

Concerning freshwater toxicity results for azoxystrobin, a high interspecific variability was also found and no statistical differences in sensitivity were attained between different trophic groups (microalgae, invertebrates and fish). The current freshwater species at most risk is the diatom *N. pelliculosa*, with an ErC_{50,120h} of 49 µg L^{-1}. The azoxystrobin concentration settled by the freshwater SSD curve as the negligible risk level to organisms is 6.8 µg L^{-1} (*Table 34*). This value is three times less protective than the one established, and abovementioned, for marine environments using the azoxystrobin LLHC_{5}, and much less protective (≈90×) when considering the Ortiva LLHC_{5}. Therefore, based on the most conservative form of the SSD approach, 100 seems to be an appropriate safety factor for azoxystrobin when extrapolating the risk to marine environments from short-term freshwater toxicity.
data. Also, despite some minor reservations with regard to parity, when comparing marine and freshwater species, a greater sensitivity of marine species to azoxystrobin was also highlighted by visually comparing both SSD curves.

The PNEC values determined by the present study allow us to conclude that PNECs obtained using different methodologies may vary and the ones based on the AF approach were two or more orders of magnitude lower than those obtained through the SSD method for azoxystrobin. Several studies corroborate this conclusion, as Jin et al. (2012) for 2,4,6-trichlorophenol and Nam et al. (2014) for gold(III) ion. Moreover, PNEC values derived from active ingredient and commercial formulation toxicity data, or from marine and freshwater toxicity data, may also vary. The following ranking of environmental protection for azoxystrobin was attained: marine Ortiva> marine azoxystrobin> freshwater azoxystrobin. In the case of PNEC values, the difference between active ingredient and commercial formulation derivations is of about three times, and the difference between marine and freshwater derivations is of about two times. This latter value is much smaller than the abovementioned safety factor of 100 resulting from LLHC₅ values, which relates the effects of azoxystrobin on marine and freshwater organisms. It should be noteworthy that this factor of 100 is not in line with the guidance document of the European Chemicals Agency for the derivation of marine no-effect levels based on freshwater data, which recommends a safety factor of 10 (ECHA, 2008).

Finally, the lowest PNEC value obtained in the present study (1.0 µg L⁻¹ derived from marine Ortiva toxicity data) should be the one considered in risk calculations for azoxystrobin. Therefore, a validation of this value by comparing it with NOEC values from single-species tests and with multi-species experiments was made. Ten long-term NOEC values derived from freshwater species, covering from aquatic fungi to fish, were reported by Rodrigues et al. (2013c), and the data ranged from 14 to >5,000 µg L⁻¹. Accordingly, the PNEC value of 1.0 µg L⁻¹ was protective against
the long-term effects of azoxystrobin, since it was always lower than the reported NOECs. Also, a recent multi-species study (outdoor chronic microcosms) presented by van Wijngaarden et al. (2014) reports a NOEC\textsubscript{population} for copepods of 1.0 µg L\textsuperscript{-1} and a NOEC\textsubscript{community} for zooplankton of 10 µg L\textsuperscript{-1}, which were obtained after the application of the commercial formulation Amistar\textregistered. Therefore, all the PNEC values derived from AF methods (PNECs ≤0.049 µg L\textsuperscript{-1}) are sufficiently protective against this important taxon. They can, however, be considered overprotective. If the more scientifically robust SSD approach is considered, the most protective PNEC value of the present study is sufficient to safeguard the copepod taxonomic group that constitutes the food and energy link between primary producers and organisms of higher trophic levels in aquatic food webs such as macroinvertebrates and fish. Their biological relevance is also explained as copepods dominate the metazoan biomass of open-water marine and freshwater environments (Turner, 2004). Therefore, after comparing the PNEC values obtained in the present study to all laboratory-derived toxicity information available for azoxystrobin, we recommend using 1.0 µg L\textsuperscript{-1} as the PNEC value for azoxystrobin in the water environmental compartment. This target value was derived from marine Ortiva toxicity data, which highlight the importance of testing commercial formulations of pesticides to attain realistic toxicity effects.
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The use of pesticides for agricultural crop protection is now a practically unavoidable fact, being the development of new chemicals an important activity within the pesticide industry. New pesticides are currently tested in a prospective way (largely supported by modelling practices) before being approved for use by regulatory agencies, and environmental concerns are taken into account in the selection criteria. In general, new active ingredients must have highly specific modes of action, low persistence and low mobility in the environment, as well as low potential for groundwater residues. Nevertheless, a balance between this billion dollar industrial activity, pesticide use and the need to ensure the safety of people, food and the environment have to be guaranteed, thus reminding us of the importance of retrospective scientific research. Therefore, during this last four years, by taking a modern and integrative approach to the occurrence, fate and effects of azoxystrobin in aquatic ecosystems, a little corner of the veil was lifted. Even though it may seem a small contribution, significant progress was achieved. For instance, validated confirmatory methods for azoxystrobin in marine complex matrices were developed. Azoxystrobin was not expected to bioaccumulate due to its low octanol-water coefficient and bioconcentration factor (Log Kow 2.5, BCF 21). However, this residue was measured in a 100% frequency in *S. plana* bivalves collected in the Mondego estuary, suggesting that predictions of the Kow and BCF may have noteworthy limitations. Also, all the six prospected pesticides (atrazine, azoxystrobin, bentazon, λ-cyhalothrin, penoxsulam and terbuthylazine) were bioaccumulated by *S. plana*, leading us to consider that pesticides may not only cause adverse effects on the aquatic organism itself, but also pose a potential
human health risk, since this is an edible species and is considered of economic interest.

This project also revealed that the invertebrate *C. maenas* showed some degree of tolerance to an environmental concentration of azoxystrobin exposure (30 µL L\(^{-1}\)). However, a noteworthy tendency was also pointed out: the combined effects of temperature and azoxystrobin unbalance the natural capability of crabs to handle a single stress. We should also highlight that, in nature, aquatic organisms are exposed to a wide variety of pesticides and to an even higher number of other substances (e.g., fertilizers, pharmaceutical and cosmetic residues, PAHs), and that climate change is expected to cause more severe climate-driven events, as was widely discussed in the 2015 United Nations Climate Change Conference held in Paris last week.

The results of the present project also revealed that pesticide toxicity could be reliably estimated by *in vitro* cell-based assays, and that the sulforhodamine B colorimetric assay is also appropriate due to its simple and inexpensive methodology. The H9c2 cell line proved to be sensitive enough to produce reliable, reproducible and robust results, for azoxystrobin toxicity was similar both *in vitro* and *in vivo*. Therefore, the present project discloses the environmental significance of cell-based assays and concludes that their application in environmental toxicity studies would greatly reduce the number of fish needed for pesticide testing without any loss of reliability, thus being able to potentially replace standard fish testing.

Our results also contributed to the hazardous assessment of azoxystrobin by setting a PNEC of 1.0 µg L\(^{-1}\) as a protective concentration value for the aquatic environmental compartment. Since this value derived from marine Ortiva® toxicity data, the importance of testing commercial formulations of pesticides to attain
realistic toxic effects was highlighted, as well as the use of marine species, since greater sensitivity to azoxystrobin was observed when compared to freshwater species. This study also showed that PNECs derived by species sensitivity distribution approaches were more reliable than those derived by assessment factor approaches.

Finally, it was possible to conclude that azoxystrobin aquatic risk characterisation in the Mondego estuary currently poses low risk to aquatic marine organisms since RQ <0.1. Moreover, RQ results based on data from Table 3 (Chapter 1), which reports 15 other concentrations of azoxystrobin measured worldwide in natural water samples (fresh and salt water), show that in five of the studies (33.3%) aquatic organisms were not protected (RQ >1), in six of the studies (40%) aquatic organisms were moderately protected (0.1 ≤ RQ <1), and only in four cases (26.7%), were organisms considered protected. Thus, in general, azoxystrobin in aquatic systems may pose a moderate risk to aquatic environments. This problem increases as, in nature, organisms are exposed to a number of different chemicals and over long periods of time.
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MAIN CONCLUSION

Before pesticides are approved by e.g., the EU or US-EPA, prospective aquatic ecotoxicological studies should be scientifically improved.

FURTHER RESEARCH

From all the areas developed in the scope of this project, one clearly leaves an open door for additional research: further contributions to the validation of an alternative assay for fish lethal testing. Due to a severe lack of data from comparative studies with sensitive cell models which test environmentally relevant hazardous substances, specific projects are required in order to accelerate the development of these new alternative testing methods, as well as achieve regulatory acceptance and implementation of in vitro assays in ecotoxicology. If successful results are achieved, a very important practical consequence may arise: the reduction of the number of fish used for aquatic toxicity testing. Let me finish by saying that this subject has everything to do with me!
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