Contribution of biochemical tools for the assessment of the ecological quality of fluvial ecosystems

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Abstract

Rivers are amongst the most threatened ecosystems in Europe. Effective mitigation and restoration actions are needed in order to prevent further degradation and to improve their ecological status. According to the Water Framework Directive (WFD; Directive 2000/60/EC), those actions are primarily based on the precision of the ecological assessment results. Indices based on communities structure are not suitable as early-warning indicators of contamination. The need for rapid and sensitive tools to reveal sub-lethal effects in aquatic organisms, able to anticipate future detrimental ecological effects, has raised interest on biomarkers as useful tools to complement the information from community structure indices. In this context, the main goal of this study was to approach the possibility of using biomarkers in different benthic macroinvertebrate taxa as a complementary tool in the assessment of the ecological status of fluvial systems. The case studies were two Northern Portuguese rivers, the Âncora (AR: 41°48'5.63"N, 8°46'28.57"W) and the Ferreira (FR: 41°11’15.06’’N, 8°27’25.47’’W) rivers, both included in the Natura 2000 Network (AR: PTCON0039, Site “Serra de Arga”; FR: PTCON0024, Site “Valongo”). In order to achieve the main goal, four tasks with specific aims were performed.

The first task consisted in a revision of studies (from 2000 to 2017) that measured biomarkers in benthic macroinvertebrates in biomonitoring programmes of fluvial systems. The literature review aimed to investigate: i) which benthic macroinvertebrate taxa are commonly used for biomarker determination in field surveys, ii) what are the most commonly assessed biomarkers in benthic macroinvertebrates and how sensitive are such biomarkers to exposure to contaminants, and iii) what are the steps forward to improve the use and the added value of combining biomarkers and community-based approaches to assess the ecological status of rivers?

The second task was conducted between July 2013 and September 2014 and aimed to assess the ecological status of two small Mediterranean rivers (AR and FR) through the analysis of benthic macroinvertebrates (North Invertebrate Portuguese Index, IPtIN) and macrophytes (Macrophyte Biological Index for Rivers, IBMR; Riparian Vegetation Index, RVI). Specific objectives were: i) to compare the performance of the two biological quality elements and the usefulness of their information for river management, and ii) to confirm adequate temporal windows to develop the monitoring surveys. Physico-chemical and hydromorphological quality elements were also monitored to support the interpretation of the biological elements assessed.

In the third task, a battery of biomarkers of neurotoxicity, biotransformation, antioxidant defences, oxidative stress and energy metabolism was seasonally assessed (autumn of 2013 and spring and summer of 2014) in different macroinvertebrate taxa collected at various sites in the AR and FR. Thirteen water physico-chemical parameters were also seasonally monitored. The concentration of seven organophosphorus pesticides and the percentage of thirty-two trace metals in sediments were determined in the spring. The main aim of this task was to investigate the potential usefulness of a battery of biomarkers evaluated in different benthic macroinvertebrate taxa to discriminate aquatic ecosystems with different levels of ecological quality and to provide further clues supporting environmental management.

The fourth task aimed to investigate the influence of anthropogenic disturbances on species richness of benthic macroinvertebrates belonging to the Chironomidae, Baetidae and Calopterygidae families. For that purpose, the metabarcoding approach was used to try to identify to species-level macroinvertebrates belonging to the previously mentioned families. These families have different tolerances to environmental disturbances, are widespread in the studied area, and are used for the calculation of the North Invertebrate Portuguese Index ($IPtI_N$) recommended by the WFD for the assessment of the ecological status of rivers (task 2). Samples were collected from FR sites with different ecological status classification (moderate, poor and bad), previously evaluated using the $IPtI_N$ index (task 2).

The complementary information given by community-based indices and biomarkers allowed to verify if it is possible to obtain an integrative procedure which is rapid, cost-effective, sensitive and capable of detecting the responses of macroinvertebrates to complex forms of pollution, conveniently reflecting cause/effect relationships, and overcoming the limitations of the established methodologies for the assessment of the ecological status of rivers.

The literature review demonstrated that biomarkers help to anticipate the detrimental effects of chemical contaminants detected in monitoring programmes. In general, studies that measured biomarkers in benthic macroinvertebrates in biomonitoring programmes of fluvial systems have been based on the use of single and tolerant species, mainly gammarids or caddisfly larvae. The most commonly used biomarkers in benthic macroinvertebrates are enzymes involved in nerve function and energy production, as well as biomarkers of biotransformation, antioxidant defences and oxidative stress. Although there is an increasing interest in the investigation of emerging contaminants, most studies focused on the so-called legacy contaminants, amongst which metals and pesticides are prominent. Therefore, further field research is required to address emerging contaminants and, in particular, to define site-specific baselines taking into

account the temporal influence of biotic and abiotic factors. Further studies including different biomarkers, environmental stressors, macroinvertebrate taxa and river types will provide the necessary information to establish more efficient and cost-effective biomarker and ecological status strategies indicative of future ecological damage.

The analysis of the physico-chemical and hydromorphological parameters recommended by the WFD, revealed that some sites of the AR and all sites of the FR presented high levels of nutrients, and that both rivers were quite altered, mainly in terms of floristic composition of the riparian communities. The evaluation of ecological quality obtained with the $IPtI_N$ was lower than that obtained with the IBMR for both rivers. Therefore, the evaluation of ecological status of rivers using only the macrophytes' responses to nutrient enrichment (IBMR) provided a partial evaluation of the effects of the stressors affecting the integrity of the river ecosystems. The RVI index contributed to a better evaluation of the ecological status of the rivers and provided also support for planning decisions regarding the management of both systems. The evaluation of the ecological status of small fluvial systems benefits therefore from an integrated multidisciplinary approach that allows a more accurate diagnosis of their ecological status. Mitigation of diffuse pollution and the restoration of the riparian zones are a priority to improve the ecological status of the studied rivers.

Regarding the biomarkers analysis, the data obtained supports the use of a battery of biomarkers in benthic macroinvertebrates to provide complementary information to diagnose ecological impairment or to establish reference sites, which is particularly useful for water authorities, to take actions preventing further deterioration of rivers´ ecological status. *Calopteryx* spp., Chironomidae and *Baetis* spp. and the spring and summer were the taxa and seasons useful for multivariate analysis, which showed distinct patterns of biological response in the three taxa. *Calopteryx* spp. and Chironomidae*,* in particular, identified distinct response patterns for the two rivers, fairly stable across seasons.

The results of the metabarcoding approach that was used to identify benthic macroinvertebrates to species-level, showed that the differences between sampling sites regarding the number of species were mainly influenced by the dipterans of the Chironomidae family. The sampling site with "poor" ecological status presented the highest species richness. This site also stood out from the other sites for having higher levels of nutrients and poorer habitat diversity in the river channel, being these important factors for macroinvertebrates with suitable to traits to colonize or persist in that site (especially chironomids).

For metabarcoding macroinvertebrates, standardized thresholds to assign taxa should be established to ensure that bioassessment results across studies can be compared.

For example, as observed in this study, changes in the similarity threshold used to cluster sequences can lead to variances in the number of different taxa found in a community. This makes it difficult to compare the results of studies utilizing different thresholds. For the success of the applicability of the DNA metabarcoding technique in benthic macroinvertebrates, it is also essential to develop robust reference libraries.

Overall, integrated results showed that the macroinvertebrate community-based approach is suitable for assigning the global ecological status of rivers. Biomarkers appear to be especially useful for providing relevant information for diagnosing and characterising the impaired health status elicited by the overall exposure to chemical and other environmental stressors, functioning as rapid and cost-effective tools capable of early indicating ecosystem disruptions. The two approaches therefore complement each other, reinforcing the need to use them in a combined way in order to foster efficient detection of pollution incidents threatening ecosystems health. The current study sets the foundations for future cost-effective biomonitoring campaigns in Mediterranean rivers, allowing to establish historical data important to understand ecosystem evolution, as well as baseline levels of diagnostic biomarkers in informative macroinvertebrate taxa.

Keywords: Macroinverterates; Macrophytes; Ecological status; River assessment; Water Framework Directive; Pollution; Biochemical biomarkers; Integrated monitoring; Neurotoxicity; Biotransformation; Oxidative stress; Energy metabolism; Diversity

Resumo

Os rios encontram-se entre os ecossistemas mais ameaçados da Europa. Ações eficazes de mitigação e restauração são necessárias, de forma a evitar uma maior degradação e melhorar o seu estado ecológico. De acordo com a Diretiva-Quadro da Água (DQA; Directiva 2000/60/EC), essas ações baseiam-se sobretudo na precisão dos resultados da avaliação ecológica. Os índices baseados na estrutura das comunidades não são adequados como indicadores de alerta precoce de contaminação. A necessidade de ferramentas rápidas e sensíveis para revelar efeitos sub-letais em organismos aquáticos, capazes de antecipar futuros efeitos ecológicos prejudiciais, aumentou o interesse dos biomarcadores como ferramentas úteis para complementar as informações obtidas pelos índices baseados na estrutura das comunidades. Neste sentido, o principal objetivo deste estudo foi abordar a possibilidade do uso de biomarcadores em diferentes taxa de macroinvertebrados bentónicos como uma ferramenta complementar na avaliação do estado ecológico de sistemas fluviais. Os casos de estudo foram dois rios localizados no Norte de Portugal, o rio Âncora (RA: 41°48'5.63"N, 8°46'28.57"W) e o rio Ferreira (RF: 41°11'15.06''N, 8°27'25.47"W), ambos incluidos na Rede Natura 2000 (RA: PTCON0039, Sitio “Serra de Arga”; RF: PTCON0024, Sitio “Valongo”). De forma a atingir o objetivo principal, foram realizadas quatro tarefas com objetivos específicos.

A primeira tarefa consistiu numa revisão de estudos (de 2000 a 2017) que mediram biomarcadores em macroinvertebrados bentónicos em programas de biomonitorização de sistemas fluviais. A revisão da literatura teve como objetivos investigar: i) quais os taxa de macroinvertebrados bentónicos comummente usados para determinação de biomarcadores em trabalhos de campo, ii) quais os biomarcadores mais comummente avaliados em macroinvertebrados bentónicos e quão sensíveis são esses biomarcadores à exposição a contaminantes e iii) quais os passos a seguir para melhorar o uso e o valor acrescentado da combinação de biomarcadores com a abordagem baseada na comunidade de macroinvertebrados na avaliação do estado ecológico dos rios.

A segunda tarefa, realiza entre Julho de 2013 e Setembro de 2014, teve como objetivo avaliar o estado ecológico de dois pequenos rios mediterrânicos (rios Âncora e Ferreira) através da análise de macroinvertebrados bentónicos (Índice Português de Invertebrados do Norte, $IPtI_N$) e macrófitas (Índice Biológico de Macrófitos de Rio, IBMR; Índice de Vegetação Ripária, RVI). Os objetivos específicos foram: i) comparar o

desempenho dos dois elementos de qualidade biológica e a utilidade das suas informações para o gestão de rios e ii) confirmar janelas temporais adequadas para o desenvolvimento das pesquisas de monitorização. Elementos de qualidade físico-química e hidromorfológica foram também monitorizados para dar suporte à interpretação dos elementos biologicos avaliados.

Na terceira tarefa, uma bateria de biomarcadores de neurotoxicidade, biotransformação, defesas antioxidantes, stresse oxidativo e metabolismo energético foi sazonalmente avaliada (outono de 2013 e primavera e verão de 2014) em diferentes taxa de macroinvertebrados bentónicos amostrados em diferentes locais do RA e do RF. Treze parâmetros físico-químicos da água foram também sazonalmente monitorizados. A concentração de sete pesticidas organofosforados e a percentagem de trinta e dois metais traço em sedimentos foram determinados na primavera. Esta tarefa teve como principal objectivo investigar a utilidade potencial de uma bateria de biomarcadores avaliada em diferentes taxa de macroinvertebrados bentônicos para discriminar ecossistemas aquáticos com diferentes níveis de qualidade ecológica e fornecer pistas adicionais que apoiem a gestão ambiental.

A quarta tarefa teve como objetivo investigar a influência de perturbações antropogênicas na riqueza de espécies de macroinvertebrados bentónicos pertencentes às famílias Chironomidae, Baetidae e Calopterygidae. De forma a atingir esse objectivo, utilizou-se a abordagem de DNA *metabarcoding* para tentar identificar ao nível de espécie os macroinvertebrados pertencentes às famílias anteriormente mencionadas. Estas famílias apresentam diferentes tolerâncias a perturbações antropogénicas, estão amplamente difundidas na área estudada e são utilizadas para o cálculo do Índice de Invertebrados do Norte ($IPtI_N$) recomendado pela DQA para a avaliação do estado ecológico de rios (tarefa 2). As amostras foram recolhidas em locais do RF com diferentes classificações de estado ecológico (razoável, medíocre e mau), previamente avaliados utilizando o índice $IPtI_N$ (tarefa 2).

A informação complementar fornecida pelos índices estruturais da comunidade de macroinvertebrados bentónicos e pelos biomarcadores, permitiu verificar se é possível obter um procedimento integrativo que seja rápido, rentável, sensível e capaz de detetar as respostas dos macroinvertebrados a formas de poluição complexas, refletindo convenientemente as relações de causa/efeito e superando as limitações das metodologias estabelecidas para a avaliação do estado ecológico de rios.

A revisão da literatura demonstrou que os biomarcadores ajudam a antecipar os efeitos prejudiciais dos contaminantes químicos detetados nos programas de monitorização. De uma forma geral, os estudos que mediram biomarcadores em macroinvertebrados bentónicos em programas de biomonitorização de sistemas fluviais têm-se baseado no

uso de uma única espécie tolerante a perturbações ambientais, sobretudo espécies da família Gammaridae ou da ordem Trichoptera. Os biomarcadores mais commumente usados em macroinvertebrados bentónicos foram enzimas envolvidas na função nervosa e produção de energia, bem como biomarcadores de biotransformação, defesas antioxidantes e estresse oxidativo.

Embora haja um crescente interesse em investigar contaminantes emergentes, a maioria dos estudos concentrou-se nos chamados contaminantes legados, sobretudo metais e pesticidas. Assim, são necessárias mais pesquisas de campo no sentido de se investigarem os contaminantes emergentes e, em particular, para se definirem linhas de base específicas locais tendo em conta a influência temporal dos fatores bióticos e abióticos. Estudos adicionais incluindo diferentes biomarcadores, estressores ambientais, taxa de macroinvertebrados e tipos de rios, irão fornecer as informações necessárias para estabelecer estratégias de biomarcadores e de estado ecológico indicativas de danos ecológicos futuros, mais eficientes e mais rentáveis.

A análise dos parâmetros físico-químicos e hidromorfológicos revelou que alguns locais do RA e todos os locais do RF apresentaram níveis de nutrientes elevados (sobretudo na primavera e no verão) e que ambos os rios se apresentaram bastante alterados sobretudo em termos de composição florística das comunidades ripárias. A avaliação da qualidade ecológica obtida com o $IPtI_N$ foi inferior à obtida com o IBMR em ambos os rios. Portanto, a avaliação do estado ecológico de rios utilizando apenas as respostas das macrófitas ao enriquecimento de nutrientes (IBMR) proporcionou uma avaliação parcial dos efeitos dos stressores que afetam a integridade dos ecossistemas fluviais. O índice RVI contribuiu para uma melhor avaliação do estado ecológico dos rios e também deu suporte para decisões de planeamento no que diz respeito à gestão de ambos os sistemas. A avaliação do estado ecológico de pequenos sistemas fluviais beneficia, portanto, de uma abordagem multidisciplinar integrada, que permite um diagnóstico mais preciso do seu estado ecológico. A mitigação da poluição difusa e a restauração das zonas ribeirinhas são uma prioridade para melhorar o estado ecológico dos rios estudados.

Relativamente à análise de biomarcadores, os resultados obtidos apoiam o uso de uma bateria de biomarcadores em macroinvertebrados bentónicos para fornecer informação complementar para diagnosticar danos ecológicos ou para estabelecer locais de referência, o que é particularmente útil para as autoridades da água tomarem ações que evitem uma maior deterioração do estado ecológico dos rios. *Calopteryx* spp., Chironomidae e *Baetis* spp. e a primavera e o verão foram os taxa e as estações do ano úteis para análises multivariadas, que mostraram padrões distintos de resposta biológica nos três taxa. Em particular, *Calopteryx* spp. e Chironomidae, identificaram

padrões de resposta distintos para os dois rios, relativamente estáveis em todas as estações.

Os resultados da abordagem *metabarcoding*, usada para identificar macroinvertebrados bentónicos a nível de espécie, mostraram que as diferenças entre os locais de amostragem relativamente ao número de espécies foram influenciadas sobretudo pelos dipteros da família Chironomidae. O local de amostragem com estado ecológico "medíocre" apresentou a maior riqueza de espécies. Este local também se destacou dos restantes por possuir maior concentração de nutrientes na água e pela menor diversidade de habitats no canal do rio, sendo estes fatores importantes para macroinvertebrados com características adequadas colonizarem ou persistirem nesse local (sobretudo os quironomídeos).

Relativamente à técnica de *metabarcoding* em macroinvertebrados, os limiares de similaridade utilizados para agrupar sequências devem ser padronizados, de forma a assegurar que resultados de bioavaliação entre estudos possam ser comparados. Por exemplo, tal como observado neste estudo, mudanças no limiar de similaridade utilizado para agrupar sequências podem levar a variações substanciais no número de taxa encontrados numa comunidade. Isso dificulta a comparação dos resultados entre estudos que utilizam diferentes limiares de similaridade. Para o sucesso da aplicabilidade da técnica de DNA *metabarcoding* em macroinvertebrados bentónicos, é também essencial o desenvolvimento de bibliotecas de referência robustas.

De forma geral, a integração dos resultados mostrou a abordagem baseada na comunidade de macroinvertebrados é adequada para classificar o estado ecológico global dos rios. A abordagem dos biomarcadores parece ser especialmente útil para fornecer informações apropriadas para diagnosticar e caracterizar o estado de saúde debilitado provocado pela exposição global a stressores químicos e outros stressores ambientais, funcionando como ferramentas rápidas e economicamente viáveis, capazes de indicar precocemente distúrbios no ecossistema. Desta forma, ambas as abordagens se complementam, reforçando a necessidade de usá-las de forma combinada, com o objetivo de se obter uma detecção eficiente dos incidentes de poluição que ameaçam a saúde dos ecossistemas. O presente estudo estabelece as bases para futuras campanhas de biomonitorização rentáveis em rios mediterrânicos, permitindo estabelecer dados históricos importantes para entender a evolução do ecossistema, bem como níveis basais de biomarcadores diagnósticos em taxa de macroinvertebrados informativos.

Palavras-chave: Macroinverterados; Macrófitas; Estado ecológico; Avaliação fluvial; Directiva Quadro da Água; Poluição; Biomarcadores bioquímicos; Monitorização integrada; Neurotoxicidade; Biotransformação; Stresse oxidativo; Metabolismo energético; Diversidade

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AChE	Acetylcholinesterase
Ag	Silver
APA	Agência Portuguesa do Ambiente
APHA	American Public Health Association
App	Apparent
AR	Âncora River
As	Arsenic
ASTM	American Society for Testing and Materials
ATP	Adenosine triphosphate
Au	Gold
Ba	Barium
BAFA	Bacterial fatty acid
BChE	Butyrylcholinesterases
BBI	Biotic Belgian Index
BI	Biological Index
bp	Base pair
BOLD	Barcode of Life Data System
BPMO	Benzo(a)pyrene monooxygenase
BQE	Biological Quality Element
Ca	Calcium
CAT	Catalase
CbE	Carboxylesterase
Cd	Cadmium
CDNB	1-chloro-2,4-dinitrobenzene
CEC	Contaminant of Emerging Concern
ChE	Cholinesterase
cm	Centimetre

Co	Cobalt
COD	Chemical Oxygen Demand
COI	Cytochrome *c* oxidase subunit I
Cond	Conductivity
Cr	Chromium
Cs	Cesium
Cu	Copper
C18	Eighteen-carbon
DCA	Detrended Correspondence Analysis
DNA	Desoxyribonucleic acid
DO	Dissolved Oxygen
DTNB	5,5′-dithiobis(2-nitrobenzoic acid)
d.w.	Dry weight
E	Pielou's Equitability index
eDNA	Environmental desoxyribonucleic acid
EN	European Norm
EPA	Environmental Protection Agency
EPT	Ephemeroptera, Plecoptera, Trichoptera
EQR	Ecological Quality Ratio
EU	European Union
Fe	Iron
FPD	Flame Photometric Detector
FR	Ferreira River
g	Gram
GBIF	Global Biodiversity Information Facility
GC	Gas Chromatography
GC – FPD	Gas Chromatography – Flame Photometric Detector
GSH	Reduced glutathione
GSSG	Glutathione disulfite
GIG	Geographical Intercalibration Group

GPx	Glutathione peroxidase
GQC	Grau de Qualidade do Canal (or Channel Quality Degree index)
GR	Glutathione reductase
GST	Glutathione-S-transferase
GSTPX	Se-independent peroxidase
G6PDH	Glucose-6-phosphate dehydrogenase
h	Hour
H'	Shannon-Wiener index
Hg	Mercury
HMS	Habitat Modification Score index
HQA	Habitat Quality Assessment index
HRMP	Hydrographic Region Management Plan
H_2O	Water Molecule
H_2O_2	Hydrogen peroxide
IBMR	Macrophyte Biological Index for Rivers
IBMWP	Iberian Biological Monitoring Working Party Index
IASPT	Iberian Average Score Per Taxon
IC	Intercalibration Exercise
IDH	Isocitrate dehydrogenase
INAG	Portuguese Water Institute
$IPtI_N$	North Invertebrate Portuguese Index
IQR	Interquartile range
IS	Internal Standard
K	Potassium
KPa	Kilopascal
kg	Kilogram
km	Kilometre
km^2	Square kilometre
kW	Kilowatt
L	Litre

LDH	Lactate dehydrogenase
$Log_{10}N$	Logarithmic of the total number of organisms, N (in base 10)
LPO	Lipid peroxidation
LOD	Limit of detection
LOQ	Limit of quantification
M	Molar
m	Metre
MDA	Malondialdehyde
ME	Malic Enzyme
MeCN	Acetonitrile
MedGIG	Mediterranean Geographical Intercalibration Group
mg	Milligram
$MgSO_4$	Magnesium sulphate
min	Minutes
mL	Millilitre
mM	Millimolar
mm	Millimetre
Mn	Manganese
Mo	Molybdenum
MS	Member-State
MSFD	Marine Strategy Framework Directive
mtDNA	Mitochondrial DNA
mtDNA COI	Mitochondrial cytochrome *c* oxidase subunit I
MΩ	Milliohm
m^3	Cubic metre
m^2	Square metre
$Na_2HCit\ 1.5H_2O$	Disodium citrate sesquihydrate
$Na_3Cit\ 2H_2O$	Sodium citrate dihydrate
NaCl	Sodium chloride
NADH	Reduced form of nicotinamide adenine dinucleotide

NaHCO3	Sodium bicarbonate
n.d.	Not determined
ng	Nanogram
NGS	Next-generation sequencing
NH_4^+	Ammonium ion
Ni	Nickel
n.i.	Not identified
nm	Nanometre
nmol	Nanomole
No	Number
NO_2^-	Nitrites
NO_3^-	Nitrates
O_2	Oxygen molecule
$O_2^{\bullet -}$	Superoxide anion radical
$OH^{\bullet}$	Hydroxyl radical
OM	Organic Matter
OTU	Operational Taxonomic Unit
P	Phosphorus
PAH	Polycyclic Aromatic Hydrocarbon
Pb	Lead
PBC	Polychlorinated biphenyl
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PCU	Platinum-Cobalt Units
Pd	Palladium
PFC	Perfluorinated Compound
PP	Polypropylene
ppm	Parts per million
PSA	Primary Secondary Amine
PSU	Practical Salinity Unit

PUFA	Polyunsaturated fatty acid
QBR	Qualitat del Bosc de Ribera (or Riparian Forest Quality index)
QuEChERS	Quick Easy Cheap Effective Rugged Safe
Rb	Rubidium
RBMP	River Basin Management Plan
RSD	Relative Standard Deviation
RHS	River Habitat Survey
ROS	Reactive Oxygen Species
RVI	Riparian Vegetation Index
S	Sulphur
s	Second
Sal	Salinity
Sb	Antimony
Sc	Scandium
SD	Stardard Deviation
Sn	Tin
SOC	Sediment Organic Carbon
SOD	Superoxide dismutase
Sr	Strontium
TBA	2-thiobarbituric acid
TBARS	Thiobarbituric acid-reactive substances
TDS	Total Dissolved Solids
Te	Tellurium
Temp	Temperature
Th	Thorium
Ti	Titanium
TOC	Total Organic Carbon
TPP	Triphenyl phosphate
Tris	Tris(hidroximetil)aminometano
TSS	Total Suspended Solids

U	Uranium
UK	United Kingdom
UV	Ultraviolet
UV-VIS	Ultraviolet-visible
V	Vanadium
W	Tungsten
WFD	Water Framework Directive
WWTP	Wastewater Treatment Plant
XRF	X-ray fluorescence
Zn	Zinc
Zr	Zirconium
x *g*	Times gravity
°C	Degree Celsius
µg	Microgram
µL	Microlitre
µm	Micrometre
µS	Microsiemens
%	Percentage
% DO	Percent saturation of Dissolved Oxygen

01

CHAPTER

General Introduction

General Introduction

1.1. Water Framework Directive

The European Commission's Water Framework Directive (WFD; Directive 2000/60/EC; EC 2000), transposed into the national legal order through Law No 58/2005 of 29 December 2000 (the Water Act) and Decree-Law No. 77/2006 of 30 March, is now the main tool for the integrated management of water resources in the European Union (INAG 2008). The WFD establishes that EU member states (MSs) shall protect, enhance and restore aquatic environment through the implementation of programmes of measures developed and implemented as part of river basin management plans (RBMPs) to maintain or achieve the "good status" target of all water bodies (groundwater bodies, rivers, lakes, transitional waters and coastal waters) by 2027 at the latest (EC 2011). As regards surface waters, a "good status" occurs when both the chemical and ecological statuses are at least "good".

The WFD requires MSs to identify river basin districts and assign a competent authority responsible for the Directive's implementation within each district. In addition, a RBMP must be developed for each river basin districts, and it should be designed to ensure that the provisions of the Directive and related European Community legislation are implemented at the basin level (Riew-Clarke and Allan 2010). In Portugal, RBMPs have been replaced by hydrographic region management plans (HRMPs), being the hydrographic region (consisting of one or more hydrographic basins and its coastal waters) the main unit of water planning and management. The competent authority responsible for drawing up the HRMPs is the Portuguese Environment Agency (APA).

The chemical status of surface water bodies is evaluated by determining the concentration of priority substances (PSs) and certain other pollutants, in accordance with the environmental quality standards (EQS) set out in Annex II of the Directive 2013/39/EU (EU 2013). This Directive defines EQS as the concentration of a particular pollutant or group of pollutants in water, sediment or biota which should not be exceeded in order to protect human health and the environment, and it is based on chronic toxicity data for annual average value and from acute toxicity data for maximum allowable concentration (EU 2013).

Under the WFD, the ecological status is defined as an expression of the quality of the structure and functioning of aquatic ecosystems associated with natural surface

waters (EC 2000). This status is assessed based on biological quality elements (BQEs), alongside the evaluation of hydromorphological and physico-chemical quality elements to aid the interpretation of results of biological assessments (EC 2000). The WFD recommends using four BQEs to assess rivers' ecological status, namely phytobenthos, macrophytes, benthic macroinvertebrates and fish. The ecological status of a water body is determined by the BQE that presents the worst classification, i.e. the most affected element by human activity. This principle is called “one out - all out” (EC 2000). The use of multiple BQEs provides a broader perspective in assessing ecological water quality, as each BQE may respond differently to specific environmental variables (INAG 2009). Although the simultaneous use of multiple BQEs has been recommended in areas where the main stressors are unknown, the costs associated with sampling and processing (e.g. taxonomic identification) are not trivial. Ideally the selection of the BQEs should be based on their greatest sensitivity to the main stressor(s) as well as on the uncertainty associated with the selected metrics (Johnson et al. 2006; Marzin et al. 2012). For instance, if the focus of the study is nutrient enrichment, phytobenthos and/or macrophytes should be considered; if the focus of the study is organic pollution, benthic macroinvertebrates and/or fish should be alternatively considered, as these groups are more directly affected by oxygen conditions. Lastly, if multiple stressors are being assessed, benthic invertebrates and/or macrophytes should be considered, as they also respond to other stress types (Hering 2006; Johnson et al. 2006). The selection of the most appropriate BQEs for monitoring hydromorphological degradation is dependent on the stream type. For example, in lowland streams and in medium- to large-sized rivers, fish, macroinvertebrates and macrophytes can be considered (Hering et al. 2006).

The classification of the ecological status is made by national assessment methods developed individually by the MSs, along with basic standards specified by the WFD, in order to include specific biological features (e.g. taxonomic composition and abundance) and to express results (given in five classes: high, good, moderate, poor and bad) as ecological quality ratios (EQRs) (Pokaine et al. 2014). The EQR is the ratio between an observed biological parameter value and an expected value under reference conditions (representing the steady state of an ecosystem in the absence of significant human disturbance) for the same water body type (EC 2011). A “good” ecological status of a surface water body is achieved when the values of the BQEs show low levels of disturbance resulting from human activity, but deviate only slightly from those normally associated with the same surface water body type under undisturbed conditions (GWP 2015). Although simple enough in theory, the EQR concept is rather difficult to put into practice in the pragmatic implementation of the WFD. It requires that several key issues are addressed, including the choice of appropriate indicators, typology, reference

conditions, and agreement on common principles for setting quality class boundaries (Van de Bund and Solimini 2007). These issues were addressed during the intercalibration exercise (IC), developed to ensure the comparability across EU countries of the classification results of the biological assessment systems. Geographical intercalibration groups (GIGs) were created for the IC purpose, and aggregate countries or parts of countries sharing common intercalibration types (EC 2011). The Mediterranean geographic intercalibration group (MedGIG) is one of these groups and includes regions/countries surrounding the Mediterranean basin and with Mediterranean climate, covering the Mediterranean areas of Portugal, Spain, France, Italy, Slovenia, Greece and Cyprus (Ferreira and Sabater 2014).

During the first phase of the IC (2003–2007), in the MedGIG (as well as in the other GIGs), it was not possible to conclude what was originally forecasted namely regarding the intercalibration of all biological elements and all defined river types. This lead to an extension of the process until 2011 – second phase of the IC (2007–2011) – so as to enable that all unanswered questions of the first phase were addressed. During the first phase it was only possible to intercalibrate the BQEs benthic macroinvertebrates and phytobenthos (EC, 2008). These indices, still of preliminary nature, were revised in the second phase of the IC (with more available monitoring data), contributing to changes in the EQR values (EC 2013). The revision process not only allowed the determination and promotion of the precision and confidence level of the classification results, but also enabled the intercalibration of the remaining BQEs in rivers, namely fish and macrophytes. The second phase was completed by a second Commission Decision in 2013, including new and updated results (EC 2013). The results of this second phase of the IC for the MedGIG are exposed in five articles of the Science of the Total Environment Journal (Aguiar et al. 2014; Almeida et al. 2014; Feio et al. 2014a,b; Segurado et al. 2014).

1.2. Main stressors affecting European rivers

The WFD's environmental objectives were to be met by 2015, provided that no deadline extension or exception was invoked (EC 2012). However, fifteen years after the Directive was introduced, about 47% of EU surface waters did not reach the "good" ecological status (EC 2012). During the first WFD cycle, which operated from 2009 to 2015, the number of surface water bodies presenting "good" status increased only by 10% (Van Rijswick and Backes 2015). Whilst single stressors such as strong organic pollution and

acidification of freshwaters are nowadays affecting just 14% and 10% of rivers respectively (EEA 2012), Europe's water bodies and water resources are now affected by a complex mixture of stressors resulting from urban and agricultural land use, hydropower generation and climate change (e.g. Schinegger et al. 2012; Stelzenmüller et al. 2010).

European rivers are mainly impacted by diffuse pollution (in particular from agriculture) and by hydromorphological pressures (due to hydropower production, navigation, agriculture, flood mitigation measures and urban development) (EC 2012, 2015). Diffuse pollution or non-point source pollution, may be defined as “all sources of pollution that enter waters other than from identifiable entry points”, and can thus encompass contaminants that enter water bodies through surface runoff or by soil percolation (Howarth 2011). Hydromorphological degradation is an even more vague term, including hydrological stress from low flows and water abstraction, flash floods, and morphological stress from barriers, straightening, bank fixation, and removal of riparian vegetation (ETC-ICM 2012). Both diffuse pollution and hydromorphological degradation are composed by several individual components with complex interactions.

There is a growing awareness of the increased pressure on riparian zones worldwide as a result of human activity (Tickner et al. 2001). The riparian zones are settled in a transition area between the terrestrial and aquatic areas, and function as ecotone systems within the landscape, with extreme importance for the lateral, longitudinal and vertical flows of energy and biomass (Naiman and Décamps 1997). These areas have important functions in streams’ integrity such as promoting lateral connectivity, bank stabilization, shading, temperature regulation, runoff control and increase of instream habitat diversity (Gurnell et al. 2005; Kiffney et al. 2004; Naiman and Décamps 1997; Tabacchi et al. 1998, 2000).

Most riparian ecosystems are considered of high ecological and economical value, and many are included in the European Habitat Directive (ECDGE 2003) due to their priority interest for conservation (Liendo et al. 2015). They are highly vulnerable to invasion by alien plants largely due to their dynamic hydrology, their role as conduits for efficient propagule dispersal, their human-driven degradation, their nutrient and water conditions and the intense disturbance regimes they experience (Crudhman and Gaffney 2010; Hood and Naiman 2010). However, human settlements and activities around rivers such as building activities (González-Moreno et al. 2013), construction of dams to regulate water flow (Catford et al. 2011, Greet et al. 2013), transport networks (Gelbard and Belnap 2003; Marcantonio et al. 2013) or even agriculture (Chytrý et al. 2008) may facilitate the growth of alien plant populations in these riparian ecosystems by modifying environmental conditions and establishing new sources of propagules in the vicinity of

these systems (Meek et al. 2010). Invasions by alien plants in riparian communities can reduce ecosystem services provided by riparian zones, affecting flood patterns, water table levels and soil moisture conditions (Meek et al. 2010; Tickner et al. 2001). Therefore, it is of utmost importance to disentangle the contributing factors of the invasion process in order to conserve and manage riparian habitats. In the Iberian Peninsula there has been progress towards the knowledge of alien flora (Almeida and Freitas 2006; Campos and Herrera 2009; Romero 2007; Sanz-Elorza et al. 2004; Verloove and Alves 2016). Some studies have focused on the assessment of plant invasion in riparian habitats (e.g. Aguiar et al. 2001, 2006, 2007; Biurrum et al. 1994, 2013; Tabacchi et al. 1996) and on their impact in these valuable ecosystems (Liendo et al. 2015).

Although the WFD can be an effective regulation to promote the restoration and ensure the ecological sustainability of aquatic resources, it is evident that a "good status" in water bodies can be difficult and time-consuming to achieve due to decades of previous degradation and persisting ineffective management (Adler 2003; Hering et al. 2010). Moreover, there is evidence indicating aquatic systems deranged by human activity may take on average 10-20 years to achieve functional recovery, with both community and ecosystem level variables responding on contemporary time scales (Jones and Schmitz 2009). The WFD therefore allows member states (MSs) to extend the initial deadline of 2015 up to 2027, such deadline extensions requiring to be supported by clear and well-reasoned justifications (EC 2000). MSs that avail themselves of an extension beyond 2015 are required to achieve all WFD environmental objectives by the end of the second and third management cycles, which extend from 2015 to 2021 and 2021 to 2027, respectively (EC 2012). At present it is difficult to estimate the percentage of water bodies that will achieve a “good status” by 2021 and 2027, as MSs seldom provide such information in River Basin Management Plans (EEA 2012).

1.3. Measurement of biomarkers in benthic macroinvertebrates

The introduction of the WFD established a new era in environmental risk assessment. In addition to incorporating the compliance of chemical quality standards, the key objective of the WFD is the general protection of the aquatic environment in its entirety (Hagger et al. 2006). Although the WFD’s monitoring programmes involves the use of both chemical

and biological parameters, the use of the effect-based tools such as biomarkers have been proposed for bridging the gap between chemical contamination and ecological status for evaluating the environmental status of rivers (Allan et al. 2006; Brack et al. 2017; Hagger et al. 2006). Biomarkers were defined by Depledge (1994) as "biochemical, cellular, physiological or behavioural variations that can be measured in tissue or body fluid samples or at the level of whole organisms that provide evidence of exposure and/or effects from one or more contaminants". Given that the ultimate goal of environmental monitoring is to protect biological/ecological systems, it is necessary and imperative to study the overall biological effects of exposure to potentially harmful substances to the environment (Lam 2009).

Indices based on community structure, currently used under the WFD to assess the ecological quality of surface waters, can only detect relevant effects that usually involve the eradication of one or several species from a particular site. Because this represents a loss of biodiversity, while having high ecological relevance they are of limited interest to anticipate specific protection measures required to maintain ecological quality or prevent its damage. The need for rapid and sensitive tools to reveal sub-lethal effects in aquatic organisms, able to anticipate future detrimental ecological effects, has raised interest on biomarkers as useful tools to complement the information from community structure indices (Allan et al. 2006; Brack et al. 2017; Hagger et al. 2006). As measured at lower levels of biological organisation, biomarker responses occur in shorter timescales. In particular, multibiomarker evaluations can provide early signs of exposure and adverse outcomes, translating the integrated impact of natural stressors and chemical contaminants to which animals are exposed (Allan et al. 2006; Hagger et al. 2006).

Aquatic organisms are usually exposed simultaneously to a wide range of chemicals (rather to individual substances) that may interact in organisms in different ways (e.g. additively, antagonistically or synergistically) (Lam 2009; Martinéz-Haro 2015). Chemical analyses can only provide information about the presence and/or concentrations of individual chemicals in the study system (Martinéz-Haro et al. 2015), which may not always be related with a toxic effect. Besides, current methods of chemical analyses are not adequate for detecting all possible pollutants and products of their transformation entering the aquatic environment (Martinéz-Haro 2015). In comparison with chemical analyses, the biomarker approach has the advantage of providing information on the early exposure and/or effects of the chemicals, including the combined effects of mixtures of compounds (both known and unknown), on living organisms. Therefore, biomarkers can offer a good link between both classical chemical

and biological approaches, as they deal holistically with the adverse consequences on health status caused by possible exposures (Capela et al. 2016).

Biotransformation and antioxidant enzymes have been amply used as biomarkers to assess the effects of contaminants on aquatic organisms (Diamantino et al. 2001; Frasco et al. 2002; Guilhermino et al. 1996; Lima et al. 2007). Biotransformation enzymes are responsible for the conversion of contaminants into more hydrophilic metabolites, and/or their conjugation with important molecules, facilitating their excretion (e.g. phase II glutathione-S-transferase – GST), and antioxidant enzymes are involved in the detoxification of reactive oxygen species (ROS) preventing oxidative damage (e.g. superoxide dismutase – SOD; catalase – CAT, glutathione peroxidase – GPx, glutathione reductase – GR). When the formation of free radicals by xenobiotic metabolism exceeds the endogenous protection (constituted by specific enzymes, antioxidant vitamins, and other scavengers), cellular damage will occur (Livingstone 2001), which in turn is frequently associated with membrane degradation. The degradation of membrane lipids due to free radical reactions (Lipid peroxidation – LPO) results in the production of compounds, such as malondialdehyde (MDA). The presence of MDA is indicative of oxidative damage and the reaction of MDA with 2-thiobarbituric acid (TBA) is one of the most widely used estimators of oxidative stress (Oakes and Van Der Kraak 2003). Inhibition of cholinesterases (ChEs) is also among the most used biomarkers of aquatic contamination (Damásio et al. 2011; Domingues et al. 2010; Kristoff et al. 2010). Acetylcholinesterase (AChE) hydrolyses the neurotransmitter acetylcholine at cholinergic synapses and neuromuscular junctions of both vertebrates and invertebrates. This enzyme is specifically inhibited by organophosphorus and carbamate insecticides but can also be affected by non-specific inhibitors like some metals, polycyclic aromatic hydrocarbons (PAHs) and even emerging contaminants such as pharmaceuticals. Its inhibition causes an over-accumulation of the neurotransmitter acetylcholine in the synaptic cleft or the neuromuscular junction, thus promoting prolonged electrical activity at nerve endings which may ultimately lead to death (Berra et al. 2006; Damásio et al. 2011; Domingues et al. 2010; Garcia et al. 2000; Payne et al. 1996; Pestana et al. 2009; Santos et al. 2012; Schulz and Liess 2000; Siebel et al. 2010). Likewise, enzymes involved in energy production have also been used as biomarkers to assess the effects of contaminants (Lima et al. 2007; Van der Oost R et al. 2003), since exposed organisms may need additional energy for detoxification to maintain homeostasis of physiological/biochemical functions (Choi et al. 2001). Lactate dehydrogenase (LDH) is a glycolytic enzyme involved in the anaerobic pathway of enegy production and considered a key enzyme in muscular physiology, particularly under conditions of chemical stress, when high levels of energy are required in short periods

of time (De Coen et al. 2001). Increased activities of LDH have been associated with increased metabolism under stressful conditions (Damásio et al. 2011; Moreira et al. 2006; Prat et al. 2013). The measurement of isocitrate dehydrogenase (IDH) activity seems to be important, due to its role in the aerobic pathway of energy production and its contribution to antioxidant responses, where it is involved in the regulation of the cell redox balance (Jo et al. 2001; Lee et al. 2002). An increase in IDH activity was previously observed in invertebrates exposed to contamination (Berra et al. 2004; Lima et al. 2007; Rodrigues et al. 2013).

Despite the development of evaluation methodologies with different BQEs, benthic macroinvertebrates have been the most used in the assessment of the ecological status of rivers in Europe (Lücke and Johnson 2009). Their use as bioindicators of water quality is due to their favourable biological and ecological characteristics for environmental monitoring studies, which also make them particularly attractive for biomarker measurement studies, such as: i) wide distribution, ii) relative abundance, iii) sensitivity to different pollutants, iv) sessile or limited migration patterns which facilitates spatial analysis of pollution effects, v) life cycles long enough to integrate and reflect the environmental quality of their habitats, vi) relatively simple sampling methodology that does not adversely affect the environment, and vii) well-described taxonomy of genus and families (Hare 1992; Metcalfe-Smith 1994).

Over the last decade, the inclusion of biomarkers in field surveys of contaminated rivers and streams using benthic macroinvertebrate species is increasingly being reported (Berra et al. 2004; Minutoli et al. 2013; Kaya et al. 2014; Olsen et al. 2001). However, studies aiming to evaluate the ecological quality of rivers using both biomarker- and community-based approaches in benthic macroinvertebrates are still rare (Barata et al. 2005; Damásio et al. 2011; Prat et al. 2013; Puértolas et al. 2010). In general, these studies have shown that the use of a battery of sensitive biomarkers of a large set of biochemical responses in local species of ecological importance may improve the capability of ascertaining the causes of a failing ecological status of a given river (Barata et al. 2005; Damásio et al. 2011; Prat et al. 2013; Puértolas et al. 2010). Most of these studies were also based on the determination of biomarkers on a single and tolerant macroinvertebrate species. Some authors, however, have found that the evaluation using single species may result in either under or over estimation of the risk, depending on the species selected (Berra et al. 2004; Bonzini et al. 2008). Therefore, a multi-biomarker- and multi-taxa approach is expected to provide a more integrative and complementary view of ecosystem health by encompassing diverse forms of biological integration of the environment, multiple exposure routes and different species'

sensitivities, and allowing for the validation of results from biomarkers' and species' evaluations (Duarte et al. 2017).

1.4. DNA-based methods as an alternative tool for morphology-based identification of benthic macroinvertebrates

In the current era of biodiversity loss, the assessment and management of anthropogenic impacts on freshwater ecosystems becomes a central challenge (Elbrecht and Leese 2017). Benthic macroinvertebrates are an important group for such bioassessments, as they are common and widespread, with high species diversity with varying sensitivity to environmental disturbances (Resh 2007; Rosenberg and Resh 1993). Although species within a higher taxonomic group may exhibit diverse responses to stress (Macher et al. 2016), in most bioassessments, benthic macroinvertebrates are identified to family- or genus-level, mainly because identifying organisms at species-level is extremely laborious, time-consuming and therefore expensive (Marshall et al. 2006). Moreover, frequent identification errors occur at species-level and several freshwater taxa simply lack morphological diagnostic characters at the juvenile (larvae, nymph) and even the adult stages (cryptic species; Cook et al. 2008; Liu et al. 2003; Weiss et al. 2014).

Taxonomic sufficiency has been commonly applied to bioassessment studies, especially in aquatic systems (reviews by Bates et al. 2007; Bowman and Bailey 1997; Jones 2008; Terlizzi et al. 2003). Reviews have generally concluded that the use of taxonomic detail has little influence in the interpretation of multivariate community data in aquatic ecosystems (Bates et al. 2007; Bowman and Bailey 1997; Terlizzi et al. 2009; Waite et al. 2004). However, other authors argue that the use of coarse taxonomic resolution can obscure patterns in bioassessment metrics and hinder detection of biological impacts. Thus, higher taxonomic resolution is benefitial to maximize the diagnostic capability of assessment tools (Hawkins 2006; Jones 2008; Pfrender et al. 2010).

DNA barcoding is a promising alternative tool for morphology-based identification of benthic macroinvertebrates and has been promoted as a way to increase taxonomic resolution and, thereby, to increase the sensitivity of bioassessment metrics (Cheessman et al. 2007; Stein et al. 2013; Waite et al 2004). Comparing with traditional morphological identification, the DNA barcoding has the advantage of being independent

of the users' taxonomic expertise and makes it possible to assign species names to specimens that are challenging (or impossible) to identify in any other way (e.g. cryptic, small and rare species). The ability to distinguish larvae of benthic macroinvertebrates such as caddisflies (Trichoptera; Hogg et al. 2009), stoneflies (Plecoptera; Zhou et al. 2009), dragonflies (Odonata; Rach et al. 2008), midges (Diptera: Chironomidae; Ekrem et al. 2007; Kranzfelder et al. 2017; Pedrosa et al. 2017), blackflies (Diptera: Simuliidae; Pramual and Wongpakam 2014; Rivera and Currie 2009) or mayflies (Ephemeroptera; Cardoni et al. 2015; Elderkin et al. 2012; Rutschmann et al. 2014; Stahls and Savolainen 2008) through barcoding, finally puts biodiversity assessments of aquatic communities in comparable terms to those used for terrestrial ecosystems, where estimations of biodiversity for plants and animals are never quantified at the level of genus or family (Sweeney et al. 2011). This method consists in assigning a specie to a specimen by amplifying and sequencing (using classical Sanger-based Sequencing) a standardized short DNA fragment and comparing it against a reference database of sequences already assigned to specific taxa and analysing the similarities between the sequence obtained and the sequence stored in the database (Hebert et al. 2003; Stein et al. 2014). The efficiency and accuracy in taxonomic identification using barcoding largely depend on the targeted barcode, which should be taxonomically informative (Liu et al. 2008), a primer set used for amplification, which should be adequate for the target species (Leray et al. 2013) and a reference database. It has been stated that species identification by DNA barcoding is as good and reliable as complete and accurate the reference database is (Wangensteen and Turon 2017). The most commonly used barcode for animals is a 658 bp section of the mitochondrial cytochrome *c* oxidase subunit I gene (mtDNA COI) (Hebert et al. 2003).

For diagnostic monitoring that uses the responses of species or genus to be more widely adopted, species identification needs to be more cost-effective, rapid and accurate (Carew et al. 2013). Barcoding has been applied in biodiversity conservation and environmental management (Taylor and Harris, 2012; Valentini et al., 2009) but the process is still quite laborious and expensive, because it requires each species to be processed individually (Cameron et al. 2006; Stein et al. 2014). In contrast, metabarcoding is a next-generation sequencing (NGS) technique that utilizes the same principle as classical barcoding. However, the analysis is extended to a community of individuals (of different species) rather than a single individual (Ji et al. 2013; Taberlet et al. 2012). When complete specimens are identified in "bulk", it is suggested to use the term DNA metabarcoding to distinguish approaches using environmental DNA from soil or water (eDNA) (Taberlet et al. 2012). This technique has already been tested and proposed in macroinvertebrates for use in freshwater biomonitoring programmes (e.g.

Carew et al. 2013; Elbrecht et al. 2017; Elbrecht and Leese 2017; Elbrecht and Steinke 2018; Emilson et al. 2017; Hajibabaei et al. 2011; Hajibabaei et al. 2012). Besides overcoming dependence on taxonomic expertise, this DNA metabarcoding allows rapid analyses of several samples and, consequently reduces monitoring costs and allows large-scale surveys to be performed (Kelly et al. 2014; Yu et al. 2012).

1.5. Case studies

The case studies of this work are two small Mediterranean rivers located in the North of Portugal – Âncora river (AR) and Ferreira river (FR). These rivers are subjected to different degrees of anthropogenic pressure and are both included in the Natura 2000 Network (AR: PTCON0039, Site “Serra de Arga”; FR: PTCON0024, Site “Valongo”).

Within the Water Framework Directive (WFD) implementation procedure, at the Member-States level, fifteen river types were defined in Portugal (mainland). Both the AR and FR belong to the “small sized streams of North” river type (catchment area: < 100 km^2), which reflects the country’s northern climate with high annual average precipitation (mean: 1190.25 mm ± 357.80), low annual average temperature (mean: 12.42 °C ± 1.26) (INAG 2008a). Both rivers flow in siliceous rocks (schist, granite), resulting in low infiltration soils, thereby favouring the superficial outflows and low mineralization (INAG 2008a).

The Âncora River (AR; Fig. 1A, B) springs from Serra de Arga, in the Viana do Castelo municipality (spring altitude: 816 m) and runs for approximately 17.91 km through a steep bedrock, before flowing directly into the Atlantic Ocean, in the Caminha municipality). The FR springs in Paços de Ferreira municipality (spring altitude: 550 m), has an approximate length of 22.30 km and joins the River Sousa in Gondomar municipality (Monteiro et al. 2005; Fig. 1A, C).

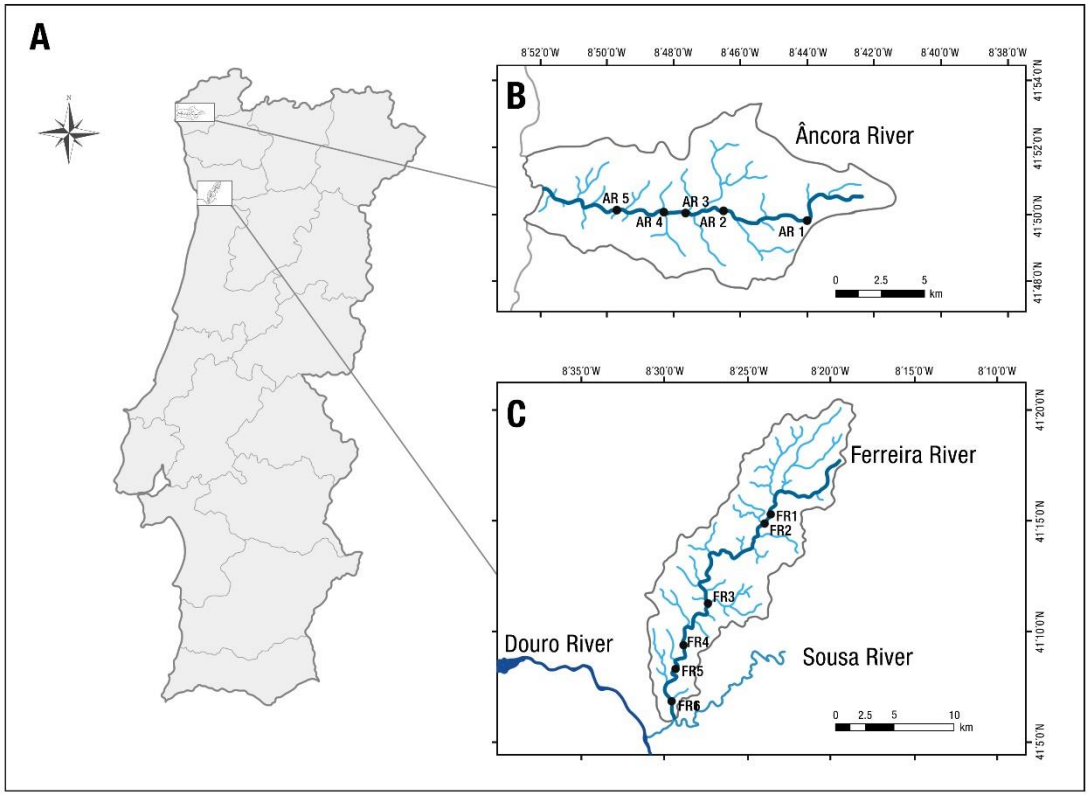

Fig. 1. Geographical situation. (A) Location of the hydrographic basins of River Âncora, B, and River Ferreira, C (rectangles), in Portugal mainland; (B) Details of the hydrographic basin of the Âncora River and the distribution of the sampling sites in the River Âncora (AR1 to AR5); (C) Details of the hydrographic basin of the Ferreira River and the distribution of the sampling sites in the River Ferreira (FR1 to FR6).

A theoretical study about the characterization of the hydrographic basins both studied rivers and their recognition through the analysis of topographic maps (military cartography 1/25.000) was conducted before selecting the final sampling sites. In that analysis, sheets nº 40 (hydrographic basin of the AR) and sheets nº 111, 123 and 134 (hydrographic basin of the FR) of the Military Charter of Portugal of the Army's Cartographic Services were used at 1/25.000 scale. Several possible sampling sites were identified and subsequently identified on *Google Earth* to obtain the GPS coordinates that would facilitate their *in situ* recognition. Then, during the spring of 2013, several field trips to both rivers allowed the selection of the final sampling sites.

Table 1.

Location of the sampling sites of the Âncora (AR1 to AR5) and Ferreira rivers (FR1 to FR6), coordinates and land use near the river banks.

Site	Location (municipality, parish)	Coordinates	Land use
AR1	Viana do Castelo, Montaria	41°47'47.95"N, 8°43'59.92"W	Woodlands, agriculture recreational área,
AR2	Viana do Castelo, Montaria	41°48'5.63"N, 8°46'28.57"W	Agriculture, recreational area
AR3	Viana do Castelo, Freixieiro de Soutelo	41°48'01.13"N, 8°47'38.72"W	Woodlands
AR4	Viana do Castelo, Freixieiro de Soutelo	41°47'59.63"N, 8°48'16.93"W	Woodlands, agriculture
AR5	Viana do Castelo, Freixieiro de Soutelo	41°48'6.22"N, 8°49'13.40"W	Woodlands, agriculture
FR1	Paços de Ferreira, Arreigada	41°15'16.34''N, 8°23'44.41''W	Urban, agriculture
FR2	Paços de Ferreira, Arreigada	41°14'58.88''N, 8°24'0.41''W	Urban, agriculture
FR3	Valongo, Campo	41°11'15.06''N, 8°27'25.47''W	Urban, agriculture, recreational area
FR4	Valongo, Campo	41°9'25.01''N, 8°29'4.49''W	Woodlands, agriculture, recreational area
FR5	Gondomar, Fânzeres e São Pedro da Cova	41°8'19.83''N, 8°29'33.50''W	Urban, woodlands
FR6	Gondomar, Foz do Sousa e Covelo	41˚6'51.35"N, 8˚29'42.62"W	Urban, agriculture

Five sampling sites in the AR (AR1 to AR5, upstream to downstream; Fig. 1B; Table 1) and six sites in the FR (FR1 to FR6, upstream to downstream; Fig. 1C; Table 1) were selected based on recommendations of national authorities for the implementation of the Water Framework Directive, such as sections of the river channel of about 100 m long representing different types of meso-habitats in terms of substrate, shading, depth, stream velocity and water movements (INAG 2008b, c). A practical criterion was also taken into account in selecting the sampling sites, namely their accessibility throughout the year (given that the physical-chemical parameters are sampled monthly). In the winter, no hydromorphological parameters were determined *in situ* nor were organisms sampled (macroinvertebrates and macrophytes) to determine community indices and biomarkers, due do the increased river river discharge at that time, making the assessment/sampling dangerous safety wise.

1.5.1. Characterization of the hydrographic basin of the Âncora River

The Âncora River (AR; Fig. 1B) is the main watercourse of the hydrographic basin of the AR, situated in the Northwest region of Portugal, belonging to the hydrographic region of Minho and Lima Rivers (PGRH1 2012). The hydrographic basin of AR covers an area of

approximately 77.5 km^2 and includes areas belonging to the municipalities of Viana do Castelo (46%) and Caminha (54%), both part of district of Viana do Castelo. Concerning land use in this basin, most of the territory (54.24%) is occupied by natural and semi-natural areas (forests, shrub and herbaceous vegetation, and open areas with little vegetation). The representativeness of artificialized areas (urban fabric, industry, trade and transport, urban green spaces, sporting, cultural and leisure areas, historic areas, etc.) and agricultural and agroforestry areas (temporary crops, permanent crops and pastures and heterogeneous agricultural areas) is 23.9% and 16.9%, respectively (PGRH1 2012). In the hydrographic basin of the AR, the main sector of activity is the primary sector (especially smallholding intensive agriculture; PGRH1 2012).

AR benefits from several protection statutes, and is almost entirely integrated into the Natura 2000 Network. The upstream section of the river is integrated in the Natura 2000 Network Site "Serra de Arga" (PTCON0039), which has an area of 4493 ha and covers the municipalities of Viana do Castelo (48%), Caminha (42%) and Ponte de Lima (10%). In Serra de Arga there are unique refuges of important wild communities, accentuating a diversified herpetofauna. The low density of human occupation, allied with soil poverty, that, in the higher areas has a large percentage of rocky outcrops, allows Serra de Arga to be in a good state of conservation. The section of the river further downstream of the AR is integrated in the Natura 2000 Network Site "North Coast" (PTCON0017), which has a total area of approximatelly 2540 ha (land area = 2048 ha, marine area = 492 ha) and covers the Atlantic coast of the municipalities of Caminha (14%), Viana do Castelo (27%) and Esposende (25%). The wide range of habitats and relevant flora and fauna aspects in both the regional and national context have justified the integration of the entire coastal strip into the National List of Sites of the Natura 2000 Network. The estuary of the AR is ecologically sensitive, of small dimensions, with some instability problems within the dynamics of the dunes located to the south, separating the marsh area from the direct contact with the sea. The riverside forest that is associated with the river is still reasonably conserved, which is of particular importance for the migration and wintering of waterfowl (DRA 1999).

Currently there is only one water abstraction station for human consumption in AR, the station of Valada, in the parish of Vila Praia de Âncora, serving some of the villages of the municipality of Caminha. The station is located upstream from Guelfa´s WWTP (41°47'56.99"N, 8°51'49.97"W), in the parish of Âncora, from the municipality of Caminha. The drainage area associated with Guelfa's WWTP extends along the coastline of the municipalities of Caminha (parishes of Vila Praia de Âncora, Âncora/Lage and Moledo) and Viana do Castelo (parish of Afife). It should also be noted that in Vila Praia de Âncora (where approximately 45% of the total population of the

hydrographic basin resides) there is a strong pressure coming from tourism, mainly in the summer months, where there are times when the floating population doubles or triples in comparison to the permanent population. Both the abstraction point and the WWTP are located downstream of the sampling sites selected for this study.

1.5.1. Characterization of the hydrographic basin of the Ferreira River

The Ferreira River (FR, Fig. 1C) is the main watercourse of the hydrographic basin of the FR located in the Northwest region of Portugal which is part of the Douro hydrographic region (PGRH3 2016). The hydrographic basin of FR has an area of area of approximately 100 Km^2, a perimeter of 83.3 Km and in its catchment area includes areas belonging to the municipalities of Paços de Ferreira (89%), Paredes (31%), Valongo (60%) e Gondomar (16%) (Monteiro et al. 2005; PGRH3 2016). Oaks, cork and holm oaks have been giving rise to eucalyptus in the last decades, mainly due to the fact that a large part of these lands are exploited by pulp companies (IEP 2003). The presence of this type of plantations dries and impoverishes the soil, also causing a strong reduction in the flow of watercourses and, consequently, biodiversity. On the other hand, several paths built during eucalyptus plantations altered the normal course of water and vegetation along the banks of some streams (Sequeira et al. 2004).

In the hydrographic basin of the FR the industrial sector predominates (e.g. furniture polishing factories, ironwork and mechanical locksmiths, slate mining, as well as product industries and metal constructions), followed by the agricultural sector (Monteiro et al. 2005). As a consequence of the increase in human population growth, accompanied by an intensification of agriculture and the strong development of industry and urbanization, the environmental quality of the Ferreira river has been deteriorated (main water line), as well as its tributaries and surrounding areas including the 2000 Network Site “Valongo” (PTCON0024) (Rodrigues 2010; Sequeira et al. 2004). This site, extending across 2553 ha, to the municipalities of Valongo (32%), Gondomar (26%) and Paredes (42%), compromises a valuable natural heritage that includes natural habitats and species of fauna and flora of conservation priority (e.g. it is the only site in continental Europe where the *Lycopodiella cernual* pteridophyte can be found, and is habitat for the lusitanian salamander - *Chioglossa lusitanica* - an endemic species of the Iberian Peninsula) (INAG 1999; Sequeira et al. 2004). Many of the species whose habitats are in this area are threatened. The greatest factors that impact biodiversity are: the pollution of the Ferreira river and its tributaries, the high urban pressure, the artificialization of

forest stands, forest fires and the introduction of exotic species (mainly acacia and eucalyptus) (Sequeira et al. 2004).

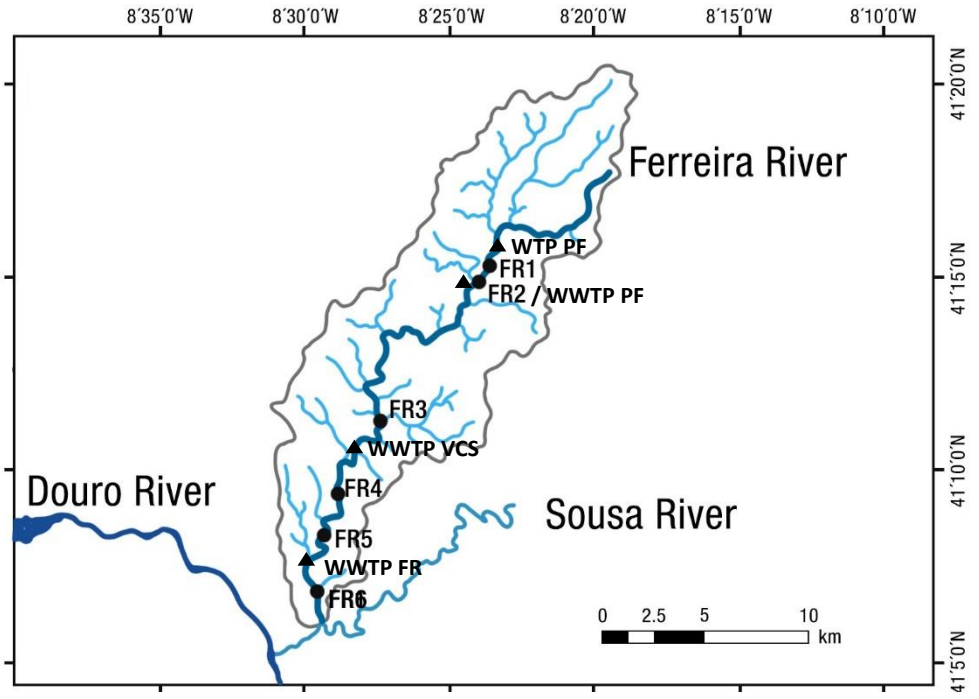

Fig. 2. Hydrographic basin of the Ferreira River with the geographic location of the existing WTP (PF: Paços de Ferreira) and WWTPs (PF: Paços de Ferreira; VCS: Valongo, Campo and Sobrado; FR: Ferreira River), as well as the distribution of the sampling sites in the Ferreira River (FR1 to FR6).

In the hydrographic basin of the FR there is one water treatment plant (WTP of Paços de Ferreira, Paços de Ferreira municipality, N41°14'58.88'', W8°24'0.41'') and three wastewater treatment plants (WWTPs), namely: i) the WWTP of Paços de Ferreira (municipality of Paços de Ferreira, parish of Arreigada, N41°14'58.88'', W8°24'0.41'', about 8.6 Km downstream of the WTP of Paços de Ferreira), ii) the WWTP of Valongo, Campo and Sobrado (municipality of Valongo, parish of Campo, N41°10'43.42'', W8°28'32.48''), and (iii) the WWTP of the Ferreira River (municipality of Gondomar, parish of São Pedro da Cova, N41°7'41.38'', W8°29'58.79'') (Fig. 2). Associated with the fact that WWTPs are not always able to meet the quality parameters related to the effluents treated, it is difficult to control some of the discharges from furniture industry or others, which together work as significant poles of pollution, impacting negatively the quality of the river waters (Monteiro et al. 2005; PGRH3 2016).

1.6. Objective and structure of the thesis

Benthic macroinvertebrates have been the most used bioindicators in the assessment of the ecological status of rivers in Europe. Indices based on benthic macroinvertebrate community structure currently used under the Water Framework Directive (WFD; EC 2000), assign a global ecological status of fluvial systems, but are of limited value as early-warning indicators of contamination.

It is now widely recognised that the WFD requires new ecological perspectives based on multidisciplinary and holistic approaches through the integration of multiple lines of evidence. In this context, the scientific community identified clear opportunities to incorporate effect-based tools such as biomarkers within the ecological approach to enhace the sensitivity and early-warning diagnosis of aquatic contamination. In this context, the main aim of this study was, through an innovative and integrative analysis, to approach the possibility of using biomarkers in different benthic macroinvertebrate taxa as a complementary approach in the assessment of the ecological status of fluvial systems, using as case studies two two small Mediterranean rivers, the Âncora and the Ferreira rivers.

This thesis is structured in six chapters. The first chapter “General Introduction”, introduces the work and explains the structure of the thesis. The sixth chapter “Main Conclusions” concerns the main conclusions of different works carried out. The other four chapters (chapters two to five) consist of individual works performed with specific aims in order to achieve the main goal:

Chapter two, “Effect-based tools for the evaluation of the ecological status of rivers: combining biomarkers and community approaches using benthic macroinvertebrates”, contributed to the understanding of the biomarkers approach in the assessment of the ecological status of fluvial systems. This chapter consists in the revision of studies from 2000 to 2017 which measured biomarkers in benthic macroinvertebrates to biomonitor chemical contaminants in fluvial systems. The focus was on studies that attempted to integrate both biomarkers and community-based approaches into the assessment of the ecological status of rivers and streams. The main goals were to answer three questions: 1) which benthic macroinvertebrate taxa are commonly used for biomarkers determination in field surveys?, 2) which are the most commonly assessed biomarkers in benthic macroinvertebrates and how sensitive are such biomarkers to the exposure to contaminants?, and 3) what are the steps forward to improve the use and the added value of combined biomarkers and community-based

approaches to assess the ecological status of rivers? The answers to those questions are important guidelines to improve future biomonitoring programs in order to achieve the best evaluation of ecosystems' health and to provide timely information so that measures can be taken before the effects become expressed at higher levels of organisation.

Chapter three, "Assessing ecological status of small Mediterranean rivers: benthic macroinvertebrates and macrophytes as complementary indicators", the ecological status of the Âncora and Ferreira rivers was assessed between July 2013 and September 2014. This work aimed at evaluating the usefulness of benthic macroinvertebrates and macrophytes in assessing the ecological status of two small Mediterranean rivers, comparing the performance of both indicators. An integrated chemical-biological effects approach was used to address the following questions: i) are the studied rivers similar in terms of physico-chemical, hydromorphological and biological parameters?, ii) which is the ecological status of these rivers, as indicated by the recently revised/intercalibrated macroinvertebrate and macrophyte indices, as well as by the macrophyte-based index of biotic integrity Riparian Vegetation Index?, iii) does temporal data support late spring as the most reliable for evaluation of the ecological status of the small Mediterranean rivers investigated?, and iv) is the quality status information provided by macroinvertebrates and macrophytes similar or complementary?

This work also allowed to verify which are the benthic macroinvertebrate taxa (sensitive and tolerant to pollution) with a greater abundance and distribution along the studied areas for the biomarkers analysis and for DNA metabarcoding.

Chapter four, "Assessing the environmental status of fluvial ecosystems employing a macroinvertebrate multi-taxa and multi-biomarker approach", aimed i) to investigate if a battery of biomarkers evaluated in different benthic macroinvertebrate taxa could discriminate aquatic ecosystems with different levels of ecological quality; ii) to understand if biomarker data can help identifying potential problems or sources of contamination affecting aquatic biota, complementing the information given by ecological quality indices, and iii) to identify the most favourable taxa and season(s) for integration of a multi-biomarker and multi-taxa analysis in cost-effective biomonitoring programmes. This study took place during the autumn of 2013 and the spring and summer of 2014, in the Âncora and Ferreira rivers. Seasonally, a battery of widely recognized biomarkers comprising a large set of biochemical responses, were assessed in different macroinvertebrate taxa. The biomarkers determined were the activity of enzymes cholinesterases (ChE), glutathione-*S*-transeferases (GST), catalase (CAT) and lactate

dehydrogenase (LDH), and the levels of lipid peroxidation (LPO). Thirteen water physico-chemical parameters were additionally measured. The amount of seven organophosphorus pesticides and the percentage of thirty-two trace metals in sediment were also determined in the spring (season of major application of pesticides in agriculture).

Chapter five, aimed to investigate the "Influence of anthropogenic disturbances on species richness of benthic macroinvertebrates belonging to the Chironomidae, Baetidae and Calopterygidae families". For that purpose, the metabarcoding approach was used to try to identify to species-level benthic macroinvertebrates belonging to the selected families. These families have different tolerances to anthropogenic disturbances, are widespread in the studied area, and are used for the calculation of the North Invertebrate Portuguese Index ($IPtI_N$) recommended by the Water Framework Directive for the assessment of the ecological status of rivers. Macroinvertebrates were collected from Ferreira River sites with different ecological status classification.

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02

CHAPTER

Effect-based tools for the evaluation of the ecological status of rivers: Combining biomarkers and community-based approaches using benthic macroinvertebrates

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Journal-Article

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Effect-based tools for the evaluation of the ecological status of rivers: Combining biomarkers and community-based approaches using benthic macroinvertebrates

2.1 Abstract

Indices based on community structure currently used under the Water Framework Directive (WFD; 2000/60/EC), provide a global ecological status of the biological communities. However, because of their limited value as early-warning indicators of contamination, biomarkers have been proposed by the scientific community to complement the ecological approach. Almost two decades after the WFD was adopted, we reviewed publications that measured biomarkers in benthic macroinvertebrates in biomonitoring programmes of fluvial systems. The focus was on studies that attempted to incorporate both biomarkers and community-based approaches into the evaluation of the ecological status of rivers and streams. Overall, from the review literature it appears that biomarker measurements of benthic macroinvertebrate species can offer complementary information on the factors threatening these communities. This information is particularly useful for water authorities, in order to take action before a system collapses into a state from which recovery is difficult or impossible, thus preventing further deterioration of the ecological status. Gaps in need to be addressed for rapid and efficient implementation of biomarkers in benthic macroinvertebrates in routine wide-scale monitoring are discussed. In particular, site-specific baselines have to be defined, taking into account the influence of biotic and abiotic factors on these biochemical responses. Further studies including different biomarkers, environmental stressors, macroinvertebrate taxa and river types, will provide crucial information on how to establish adequate biomarker strategies to indicate future ecological damage.

Keywords: Biochemical biomarkers; Pollution; Integrated monitoring; Neurotoxicity; Biotransformation; Oxidative stress

2.2. Introduction

Water resources are a key element in the balanced development of any region. However, increasing urbanisation and significant technological and industrial development, especially in the second half of the twentieth century, have resulted in an increasing water demand and the discharge of increasing quantities of chemical substances into the environment, particularly in surface waters (Vieira 2003).

Freshwater ecosystems are among the most threatened in the world (Dudgeon et al. 2006), although they support a significant part of the most biologically rich and diverse habitats (Gioria et al. 2010). Political decision-makers have recognised a range of threats to freshwater biodiversity, including chemical pollution, degradation of habitat quality, colonisation by invasive species, modification of the hydrological regime and over-exploitation of biological (e.g. fisheries) and physical (e.g. water abstraction) resources (Hill et al. 2016). In order to tackle this problem, new legislation at the European level such as the Water Framework Directive (WFD; EC 2000), has called for an evaluation of the water bodies' ecological status through integrative ecosystem approaches using biological quality elements.

Indicators of environmental quality to support and improve monitoring schemes and management performance have evolved considerably over recent decades, increasingly integrating biological measures at different levels of organisation to enhance the sensitivity and early-warning diagnosis of aquatic contamination (Adams 2002; Borja and Dauer 2008). Biomarkers provide evidence of exposure to and/or effects of one or more contaminants through behavioural, biochemical, cellular or physiological changes that can be measured in body fluid or tissue samples, or even on the whole organism (Depledge 1994). In fact, biomarker responses are now compulsory indicators of health status in key management strategies and policies, such as the Marine Strategy Framework Directive (MSFD; Directive 2008/56/EC; EC 2008). Although effect-based tools such as biomarkers are not incorporated in the WFD, there is a growing number of studies aiming at assessing the environmental impact of contaminants in European freshwater ecosystems, which integrate both community and biomarker approaches in fish (Colin et al. 2016; Damásio et al. 2007; Dietze et al. 2001; Echeverría-Sáenz et al. 2012; Mayon et al. 2006) and benthic and planktonic invertebrate species (Barata et al. 2007; Damásio et al. 2008, 2011a; Prat et al. 2013; Puértolas et al. 2010). These studies suggest that biomarkers may complement the information from community structure indices for identifying potential contamination problems affecting aquatic biota. They can provide evidence of effects that will only be detectable later, at higher levels of biological

organisation (i.e. population, community or ecosystem). Because their responses occur in much shorter time scales than those occurring at the community and ecosystem levels, biomarkers have thus been recommended for bridging the gap between chemical contamination and ecological status. They may be particularly useful for evaluating the environmental status of rivers, as they can cover a broad range of exposure routes and toxicity mechanisms in a variety of organisms (Brack et al. 2017). Furthermore, they can take into account the additional risks posed by unidentified compounds present in affected systems, and the complex mixtures of toxicants typically found (Brack et al. 2017).

2.3. Aims and Scope

The present review aims to answer three main questions: 1) which benthic macroinvertebrate taxa are commonly used for biomarker determination in field surveys? 2) what are the most commonly assessed biomarkers in benthic macroinvertebrates and how sensitive are such biomarkers to exposure to contaminants? and 3) what are the steps forward to improve the use and added value of combined biomarkers and community-based approaches to assess the ecological status of rivers?

The review cover studies from 2000 to 2017 which measured biomarkers in benthic macroinvertebrates to biomonitor chemical contaminants in fluvial systems. The focus was on studies that attempted to integrate both biomarkers and community-based approaches into the assessment of the ecological status of rivers and streams. Different information sources were used to obtain relevant literature: i) Scopus (http://www.scopus.com/), ii) Web of Science (www.webofknowledge.com/) databases and iii) European Union reports and documentation from environmental agencies, namely the United States Environmental Protection Agency (US EPA), the Canadian Environmental Assessment Agency and the European Environmental Agency (EEA). Most relevant literature was identified through Scopus and the Web of Science databases. The search terms used were "biomarkers", "macroinvertebrates" and "rivers", and included studies providing detailed indications of the benthic macroinvertebrate indices determined and/or taxa used.

2.4. Water Framework Directive

2.4.1. Good environmental status of European rivers

The main goals for the Water Framework Directive are the protection and restoration of all types of natural waters to achieve a “good status” target by 2027 at the latest, while ensuring the ecosystems’ balance by preventing deterioration and adapting nature conservation strategies to use natural resources sustainably (EC 2000). As regards surface waters, a "good status" occurs when both the chemical and ecological statuses are at least "good”. To reach the “good status” goal, Annex V of the WFD refers to three basic types of surface water monitoring: i) surveillance monitoring, to evaluate the overall status within a catchment or sub-catchment and to select the locations for operational monitoring; ii) operational monitoring, to assess the status of the water bodies identified as at risk of failing the Directive’s environmental objectives and to assess the changes in the status of the water bodies after programs of measures are applied; and iii) investigative monitoring, to ascertain the causes of a “failing” water body (if not known) or to ascertain the magnitude and impacts of accidental pollution (EC 2000). When water bodies achieve a “good status”, only surveillance monitoring is needed to ensure their maintenance, while for those which are identified as being at risk, or are of moderate or poor quality, further information (operational and investigative monitoring) is required so that water authorities can take appropriate action to improve the quality until a “good status” is achieved (Allan et al. 2006; Birk et al. 2013).

Cost-effective resource management should focus on mitigating the effects of the most harmful stressor(s) threatening ecological quality. European rivers are mainly impacted by diffuse pollution (in particular from agriculture), and by hydromorphological degradation (mainly attributable to agriculture, hydropower, flood protection, navigation and urban development) (EC 2012, 2015). Diffuse pollution or nonpoint source pollution may be defined as “all sources of pollution that enter waters other than from identifiable entry points”, and can thus encompass contaminants that enter water bodies through surface runoff or by percolation through soil’ (Howarth 2011). Hydromorphological degradation is an even more vague term, including hydrological stress from low flows and water abstraction and flash floods, and morphological stress from barriers, straightening, bank fixation and removal of riparian vegetation with a subsequent increase in water temperatures (ETC-ICM 2012). Both diffuse pollution and

hydromorphological degradation are composed of several individual components with complex interactions.

Chemicals used in agriculture are inherently toxic to non-target aquatic organisms, thus producing a wide range of impacts on the aquatic biota (Bonzini et al. 2008). Their persistence in aquatic systems varies and can occur over short periods of time, depending on the chemical group to which they belong, as well as several biotic (e.g. microbial communities) and abiotic (e.g. light) factors. In addition to pesticides, other chemicals used in medicine, industry and even common household appliances are also constantly introduced into the aquatic environment (De Castro-Català et al. 2015; Pinheiro et al. 2017; Sousa et al. 2018). Their main source is from wastewater treatment plants (WWTP) (Barbosa et al. 2016a; Oller et al. 2011) because secondary and tertiary treatment processes do not completely remove organic pollutants not having been designed specifically for that purpose (Brun et al. 2006; Deblonde et al. 2011; Gómez et al. 2007; Larsen et al. 2004; Tauxe-Wuersch et al. 2005). The uncontrolled discharge of organic substances into the environment, even at trace concentrations (i.e. ng L^{-1} – µg L^{-1}, known as micropollutants) contributes to accumulations of some of them in aquatic compartments, with potentially detrimental effects on both aquatic ecosystems and human health (Barbosa et al 2016b; Sousa et al. 2018). However, still little has been published on the ecotoxicological effects of organic micropollutants on freshwater macroinvertebrates, even though some studies have reported their presence and amount in both effluents and receiving waters (Freitas et al. 2015; Liu et al. 2018; Sousa et al. 2018).

Based on the presence or absence of regulations, the organic pollutants are classified as priority substances (PSs) or contaminants of emerging concern (CECs). The chemical status of surface water bodies is evaluated by determining the concentration of 45 priority substances (PSs), i.e. substances presenting a significant risk to or via the aquatic environment, and 8 other pollutants in accordance with the Environmental Quality Standards (EQS) based on chronic toxicity data for the annual average value and from acute toxicity data for the maximum allowable concentration, as set out in Annex II of the Directive 2013/39/EU (EU 2013). PSs comprise 41 organic pollutants – including pharmaceuticals, pesticides, dioxins and dioxin-like compounds, an organotin compound, industrial compounds, perfluorinated compounds (PFCs), polycyclic aromatic hydrocarbons (PAHs) and organic solvents – and 4 metals, namely cadmium, lead, mercury and nickel. Houtman (2010) classified CECs into three large groups of compounds: 1) compounds recently introduced to the environment (e.g. recently developed industrial compounds), 2) compounds that have only recently been detected using improved analytical techniques, despite being present in the environment

for a long time, and 3) compounds known to be present in the environment for a long time but only recently recognised as potentially causing adverse effects on ecosystems or humans (e.g. hormones). A watch list of 17 CECs (including 5 pharmaceuticals, 2 natural hormones, 8 pesticides, a UV filter and an antioxidant commonly used as a food additive) for EU monitoring was specified in Decision 2015/495/EU (EU 2015) in order to identify future control priorities through the EQS regime under the WFD.

Although the WFD can be an effective regulation to promote the restoration and ensure the ecological sustainability of aquatic resources, it is evident that a "good status" in water bodies can be difficult and time-consuming to achieve due to decades of previous degradation and persisting ineffective management (Adler 2003; Hering et al. 2010). Moreover, there is evidence indicating aquatic systems deranged by human activity may take on average 10-20 years to achieve functional recovery, with both community and ecosystem level variables responding on contemporary time scales (Jones and Schmitz 2009).The WFD therefore allows member states (MSs) to extend the initial deadline of 2015 up to 2027, such deadline extensions requiring to be supported by clear and well-reasoned justifications (EC 2000). At present it is difficult to estimate the percentage of water bodies that will achieve a “good status” by 2021 or 2027 (the end of the second and third management cycles specified by the WFD, respectively), as MSs seldom provide such information in River Basin Management Plans (EEA 2012).

2.4.2. Ecological status of rivers

Under the WFD, the ecological status is defined as an expression of the quality of the structure and functioning of the aquatic ecosystems associated with natural surface waters (lakes, rivers, transitional and coastal waters), and is classified according to biological quality elements (BQEs) as well as physico-chemical and hydromorphological quality elements to support the interpretation of the biological assessment results (EC 2000).

By integrating the complex and cumulative effects of different stressors (from point and diffuse pollution to hydrological changes, physical modifications of aquatic or riparian habitats), biological communities can be used to quantify the combined effects of these impacts (EC 2000). As they integrate environmental conditions over long periods of time, aquatic communities – especially if sessile organisms or organisms of limited migration patterns are used as bioindicators – allow the biological evaluation to be used quite efficiently to detect both intermittent acute peaks and long-term discharges of toxic

substances. Because aquatic communities are sensitive to pollution, they allow the identification of areas subject to point and nonpoint sources of contamination, even when distant from the emission source (Metcalf 1989). Chemical measurements alone only enable the quantification of emissions from human activities into water resources which are present at the time of sampling (Bernardino et al. 2000). They therefore require a large number of analytical determinations aimed at efficient time monitoring (Metcalfe 1989).

Taking into account the great diversity of pollutants and their impacts, environmental monitoring should involve an integrated weight of evidence approach from the analysis of the physical, chemical and biological quality of the water body as well as the structural quality of the habitat, in order to obtain a more complete spectrum of information for an adequate management of surface water resources. Biological methods detect and assess the degree of ecological imbalance, and physical and chemical methods are essential to identify and quantify the concentration of pollutants potentially responsible for this situation. While physical and chemical parameters are required to establish limits for regulating the actual and authorised use of waters, biological methods more accurately reflect a wide range of disturbances and impact gradients in aquatic habitats than do individual chemical quality elements. Both the quality and quantity of available habitats affect the structure and composition of resident biological communities, habitat degradation being one of the factors that most reduces the ecological quality of rivers. The WFD therefore requires the determination of riverine hydromorphological quality, in which channel patterns, variation in depth and width, flow conditions, substrate composition and structure of the riparian zone must be included in the ecological status assessment (Barquín et al. 2011). Impact of unknown interactions among the chemicals present, which can elicit synergistic detrimental effects, can only be detected through measurement of biological responses, further highlighting the importance of their use.

2.4.2.1. Biological quality elements and monitoring schemes

Biological indices (BIs) based on community structure are currently used to assess the ecological status of rivers. The WFD defines fish, phytobenthos, macrophytes and benthic macroinvertebrates as the biological quality elements (BQEs) for the category “rivers” (EC 2000). Biological assessment results need to be expressed as ecological quality ratios (EQR). The EQR is the ratio between an observed biological parameter value and an expected value under reference conditions (representing the steady state

of an ecosystem in the absence of significant human disturbance) for the same type of water body. The intercalibration exercise (IC) is used to ensure the comparability of the classification results of the biological assessment systems among EU countries by addressing several key issues (setting general quality class boundaries, choice of appropriate indicators, reference conditions, and typology) necessary to establish the EQR concept (EC 2011; Van de Bund and Solimini 2007).

A multiplicity of anthropogenic impacts are generally involved in fluvial ecosystems at the same time and, due to concomitant effects, they change the biological communities in different ways than a single impact (Piggott et al. 2012). Since each BQE has its own particular characteristics it may respond differently to specific environmental variables (INAG 2009). The use of multiple BQEs can therefore help to distinguish the effects of anthropogenically induced stress more effectively (detection of the effects of multiple stressors) and with less uncertainty (Hering et al. 2006a; INAG 2009).

The WFD states that, for the ecological status assessment during surveillance monitoring, all biological elements must be monitored at each monitoring site (EC 2000). However, the use of multiple BQEs may be seen as an unjustified increase in monitoring effort and cost, as the responses of BQEs are often correlated (i.e. redundant) (Hering et al. 2006b) and only a few studies in European rivers have compared the diagnostic value of BIs using more than two taxa (e.g. Hering et al. 2006b; Hughes et al. 2009; Johnson et al. 2006a, b; Marzin et al. 2012; Turunen et al. 2016). Ecotoxicological monitoring and assessment methods may also be appropriate (Collins et al. 2012). For example, biomarkers may help MSs to anticipate specific pollution problems and/or identify them where water bodies appear to have a good chemical status but at the same time display a bad ecological status (Sanchez and Porcher et al. 2009).

2.5. Biomarkers determination in benthic macroinvertebrates used in river biomonitoring

2.5.1 Benthic macroinvertebrates commonly used for biomarkers determination

Benthos are organisms inhabiting the bottom of aquatic ecosystems for at least part of their life cycle and associated with diverse types of substrate, both organic and inorganic (Rosenberg and Resh 1993). According to the common definition of macroinvertebrates

given by Rosenberg and Resh (1993), this category covers invertebrate fauna retained by mesh-sizes of 500 µm or less. Despite the development of evaluation methodologies with different BQEs, benthic macroinvertebrates have been the most used in the assessment of the status of European rivers (Lücke and Johnson 2009). Their use as bioindicators of water quality is due to their favourable biological and ecological characteristics for environmental monitoring studies (Hare 1992; Metcalfe-Smith 1994), which make them particularly attractive for biomarker measurement studies, such as: i) wide distribution, ii) relative abundance, iii) sensitivity to different pollutants, iv) sessile or limited migration patterns which facilitates spatial analysis of pollution effects, v) life cycles long enough to integrate and reflect the environmental quality of their habitats, and vi) relatively simple sampling methodology that does not adversely affect the environment, vii) well described taxonomy of genus and families. Thus, evaluating the community of benthic macroinvertebrates present at a given site is considered favourable for environmental monitoring studies due to their association with the sediment, which is the repository of most of the contaminants. Furthermore, they are a major food resource for fish and one of the most important constituents of fluvial ecosystems (Berra et al. 2004). Contaminants accumulated in macroinvertebrates are very likely to be transferred throughout the aquatic food web, with ensuing toxicological effects (Baird and Burton 2001).

Benthic macroinvertebrates, such as insect larvae (of different orders, e.g. Diptera, Ephemeroptera, Plecoptera, Trichoptera and Odonata), crustaceans (of the orders Isopoda and Amphipoda), molluscs (order Dreissenidae) and Oligochaeta, have already been used in ecotoxicological methods for the assessment of contaminants in lotic ecosystems (Tables 2, 3, 4). Amongst freshwater benthic macroinvertebrate species, however, gammarids (Crustacea, Amphipoda) and caddisfly larvae (Insecta, Trichoptera) have been preferentially chosen for biomarker determination in field surveys (Tables 2, 3, 4).

Gammarids are ecologically relevant species, representing an important food resource for amphibians, birds, fish and even other macroinvertebrate species (Friberg et al. 1994; MacNeil et al. 2002; Welton 1979). As they are involved in detritivorous pathways they can have a marked influence on the processing of dead organic matter in rivers (Wallace and Webster 1996). Any stressor that compromises the population viability of any of these organisms is therefore more likely to affect the whole ecosystem (Amiard-Triquet et al. 2012). These organisms are also excellent bioindicators, being common in western European streams, where they are often found at high density (Maltby et al. 2002). In addition, they have a relatively short generation time and high reproductive rate, and are highly sensitive to a range of contaminants (Peschke et al.

2014), particularly from wastewater (Peschke et al. 2014; Schirling et al. 2005; Schneider et al. 2015). Several *in situ* and mesocosm studies have used gammarids to assess the toxicity of effluents and river waters by measuring toxicity endpoints such as growth, reproduction and genotoxicity (e.g. Bundschuh and Schulz 2011; Coulaud et al. 2015; Lacaze et al. 2011).

Caddisfly larvae are considered good model species for use in ecotoxicological studies for the assessment of pesticides, detergents, pharmaceuticals and metal pollution in lotic ecosystems (Berra et al. 2006; Damásio et al. 2011a; Pestana et al. 2014; Schulz and Liess 2000; Xuereb et al. 2007). The caddisfly species commonly selected in field studies for biomarker analysis is the tolerant *Hydropsyche exocellata* (e.g. Barata et al. 2005; Damásio et al. 2011a; Prat et al. 2013; Puértolas et al. 2010). This species primarily feeds by filtering fine particles (below 1 mm diameter; Tachet et al. 2002), is large (20–100 mg wet weight) and is widely distributed, occurring in both unaltered and degraded benthic communities (Bonada et al. 2004). Specimens of the last larval instar can be collected throughout the year as the life cycle of these organisms is variable (about one year), with two to several generations per year (Barata et al. 2005).

2.5.2. Commonly measured biomarkers in benthic macroinvertebrates

Biochemical biomarkers are sensitive tools that can be determined using simple and standardised procedures (Van der Oost et al. 2003) and can be measured in different organisms (fish, mammals, molluscs, plants, crustaceans and insects). The quantification of biomarkers has been widely used for the evaluation of the effects caused by xenobiotics, both *in vitro* and *in vivo* (Binelli et al. 2006). Most biomarkers analysed in benthic macroinvertebrates are biochemical biomarkers determined under laboratory-controlled conditions in which organisms are exposed to varied concentrations of selected chemicals (De Coen et al. 2001; Hyne and Maher 2003; Kheir et al. 2001). These experiments allow the establishment of the cause and effect relationships of specific contaminants and biomarkers, and the extrapolation of the risk of such contaminants to natural populations (Clements 2000). Certain questions need to be considered for an ecological risk assessment, in order to select the most relevant biomarkers. These are: is the biomarker sensitive and easy to measure; and does it respond to the contaminant in a dose- or time-dependent manner and, if so, how long after exposure does the response lasts (Hagger et al. 2006)? Moreover, biomarkers used

in environmental biomonitoring studies should only respond to contaminants. In reality, however, they are influenced by intrinsic biotic (e.g. sex, size and reproductive status) and environmental factors (e.g. seasonality, water physico-chemical characteristics) (Jha 2008; Sanchez et al. 2008; Wiklund and Sundelin 2004). These factors complicate data interpretation because biomarker responses may be related to other factors than contaminant exposure, or to an interaction between contamination and environmental factors (Sheehan and Power 1999). In order to avoid this type of misinterpretation, in field studies it is advisable to use biomarkers in sites with similar physical and chemical parameters (Flammarion and Garric 1997). The establishment of a robust baseline, taking into account the temporal variation in biotic and abiotic factors, significantly improves the integration of biomarkers in biomonitoring approaches. Environmental factors which may influence biomarker responses should always be monitored along with the collection of organisms for biomarker assessment, so that potential confounding effects, including seasonality, can be included in integrated multivariate analysis (Cajaraville et al. 2000).

Another point to note is that, in a variety of aquatic invertebrates, some widely-used enzyme biomarkers can vary in tissue location and activity within individuals (Hyne and Maher 2003). In some fish and bivalve species, AChE activity assays have been carried out on individual tissues (e.g. fish muscle and brain, abdominal muscle of crustaceans, adductor muscle and gills of bivalve molluscs), enabling the use of homogenous samples to reduce the variability in enzyme measurements (Xuereb et al. 2009). For example, studies that determined at biomarkers in zebra mussels (Damásio et al. 2010; Faria et al. 2010a, b), measured B-esterases in gills (innervated tissues related to food intake) due to their role in filtering; and other biomarkers related to biotransformation (GST), antioxidant defences (SOD, CAT, GPx), oxidative stress (LPO) and energy metabolism (LDH) were measured in the digestive gland. A few field studies using caddisfly larvae (Damásio et al. 2011a; Prat et al. 2013; Puértolas et al. 2010) and gammarids (Maltby and Hills 2008) as sentinel species measured AChE activity in the head and other biomarkers (antioxidant and biotransformation enzymes, tissue oxidative damage markers) in the body. Insect larvae heads have several tissues rich in neuronal cells (eyes, mouth, muscles), thus having higher AChE activity than the remaining body parts (Damásio et al. 2011a). However, it is generally impractical and unrealistic to dissect specific tissues in small invertebrate species (such as insect larvae) when trying to obtain reproducible measurements using a large set of biomarkers (Xuereb et al. 2009). The majority of laboratory and field studies have therefore chosen to measure biomarkers on the whole body of benthic macroinvertebrates selected as sentinel

species (e.g. Barata et al. 2005; Berra et al. 2004; Bonzini et al. 2008; Minutoli et al. 2013; Xuereb et al. 2009).

There are increasing numbers of reports on the inclusion of biomarkers in field surveys of rivers and streams using benthic macroinvertebrate species. Most of these studies were carried out in European countries (e.g. Berra et al. 2004; Kaya et al. 2014; Minutoli et al. 2013; Olsen et al. 2001). However, biomarker measurements have only been used to determine the ecological status of rivers and streams in a few cases (Barata et al. 2005; Damásio et al. 2011a; Prat et al. 2013; Puértolas et al. 2010).

The biomarkers most commonly measured in benthic macroinvertebrates in biomonitoring programmes have been both exposure and/or effect biomarkers: i) enzymes involved in neurotransmission and energy production, mainly acetylcholinesterase and lactate dehydrogenase, respectively, ii) antioxidant enzymes (especially superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase and glutathione), and iii) biomarkers of oxidative damage to cell macromolecules, mainly lipid peroxidation and DNA damage (Tables 2, 3, 4).

Table 2.

Studies evaluating rivers and streams' ecological quality status based on neuromuscular parameters, biomarkers of biotransformation and structural indices using the benthic macroinvertebrate community (when determined).

Country	Macroinvertebrate species	Order (family)	Biomarkers	Structural indices	References
Neuromuscular parameters					
Spain	*Hydropsyche exocellata*	-Trichoptera (Hydropsychidae)	ChE, CbE	-IBMWP, -IASPT	Damásio et al. 2011a
Spain	*Hydropsyche exocellata*	-Trichoptera (Hydropsychidae)	AChE, CbE	-iMMi-T multimetric index	Puértolas et al. 2010
Spain	*Dreissena polymorpha*	-Veneroida (Dreissenidae)	ChE, CbE	n.d.	Faria et al. 2010a
Spain	*Corbicula fluminea*	-Veneroida (Cyrenidae)	AChE, CbE, PChE	n.d.	Damásio et al. 2010
Italy	n.i.	Gammaridae, Asellidae, Hirudinea, and Oligochaeta	AChE	n.d.	Bonzini et al. 2008
Italy	*Serratella ignita*	-Ephemeroptera (Ephemerellidae)	AChE	n.d.	Minutoli et al. 2013
Italy	n.i.	-Diptera (Chironomidae, Tabanidae, Tipulidae), -Plecoptera (Perlidae, Leuctricidae) -Trichoptera (Hydropsychidae, Rhyacophilidae), -Ephemeroptera (Ephemerellidae, Heptageniidae, Baetidae), -Odonata (Gomphidae, Coenagrionidae) -Amphipoda (Gammaridae), -Oligochaeta class (Lumbricidae)	AChE	n.d.	Berra et al. 2004
England	*Gammarus pulex*	-Amphipoda (Gammarridae)	ChE	n.d.	Maltby and Hills 2008
England	*Chironomus riparius*	-Diptera (Chironomidae)	AChE	n.d.	Olsen et al. 2001
Biotransformation					
Spain	*Hydropsyche exocellata*	-Trichoptera (Hydropsychidae)	GST	-IBMWP	Barata et al. 2005
Spain	*Hydropsyche exocellata*	-Trichoptera (Hydropsychidae)	GST	-IBMWP, -IASPT	Damásio et al. 2011a
Spain	*Hydropsyche exocellata*	-Trichoptera (Hydropsychidae)	GST	-ICM-Star (multimetric index), -iMMi-T (multimetric index)	Prat et al. 2013
Spain	*Hydropsyche exocellata*	-Trichoptera (Hydropsychidae)	GST	-iMMi-T multimetric index	Puértolas et al. 2010
Spain	*Dreissena polymorpha*	-Veneroida (Dreissenidae)	GST	n.d.	Faria et al. 2010a
Spain	*Dreissena polymorpha* *Corbicula fluminea* *Psilunio littoralis*	-Veneroida (Dreissenidae, Cyrenidae, Unionidae)	GST		Faria et al. 2010b
Spain	*Corbicula fluminea*	-Veneroida (Cyrenidae)	GST	n.d.	Damásio et al. 2010
Italy	n.i.	Gammaridae, Asellidae, Hirudinea, and Oligochaeta	GST	n.d.	Bonzini et al. 2008
Italy	n.i.	-Diptera (Chironomidae, Tabanidae, Tipulidae), -Plecoptera (Perlidae, Leuctricidae) -Trichoptera (Hydropsychidae, Rhyacophilidae), -Ephemeroptera (Ephemerellidae, Heptageniidae, Baetidae), -Odonata (Gomphidae, Coenagrionidae) -Amphipoda (Gammaridae), -Oligochaeta class (Lumbricidae)	GST	n.d.	Berra et al. 2004
England	*Gammarus pulex*	-Amphipoda (Gammarridae)	GST	n.d.	Maltby and Hills 2008
England	*Chironomus riparius*	-Diptera (Chironomidae)	GST	n.d.	Olsen et al. 2001

Biomarkers: AChE, acetylcholinesterase; CbE, carboxylesterase; ChE, cholineterases; GST, glutathione-S-transferase; PChE, propionylesterase. *Indices*: IBMWP, Iberian Biomonitoring Working Party index; IASPT, Iberian Average Score Per Taxon. n.i.: not identified at species level; n.d.: not determined.

Table 3.

Studies evaluating rivers and streams' ecological quality status based on biomarkers related to energy metabolism and fatty acid and biomarkers of general stress response and structural indices using the benthic macroinvertebrate community (when determined).

Country	Macroinvertebrate species	Order (family)	Biomarkers	Structural indices	References
Energy metabolism					
Spain	*Hydropsyche exocellata*	-Trichoptera (Hydropsychidae)	LDH	-ICM-Star (multimetric index), -iMMi-T (multimetric index)	Prat et al. 2013
Spain	*Hydropsyche exocellata*	-Trichoptera (Hydropsychidae)	LDH	-iMMi-T multimetric index	Puértolas et al. 2010
Italy	n.i.	-Diptera (Chironomidae, Tabanidae, Tipulidae), -Plecoptera (Perlidae, Leuctricidae) -Trichoptera (Hydropsychidae, Rhyacophilidae), -Ephemeroptera (Ephemerellidae, Heptageniidae, Baetidae), -Odonata (Gomphidae, Coenagrionidae) -Amphipoda (Gammaridae), -Oligochaeta class (Lumbricidae)	LDH, IDH	n.d.	Berra et al. 2004
Fatty acid biomarkers					
Norway	*Nemoura cinerea* *Nemurella pictetii* (Simuliidae n.i.)	-Diptera (Simuliidae), -Plecoptera (Nemouridae)	- PUFA and BAFA	n.d.	De Wit et al. 2012
Russia	n.i.	-Trichoptera -Ephemeroptera -Diptera (Chironomidae)	- PUFA in total lipids, triacylglycerols and polar lipids	n.d.	Sushchik et al. 2003
Biomarkers of general stress response					
Germany	*Gammarus pulex* *Gammarus roeseli*	-Amphipoda (Gammaridae)	-analysis of 70kD heat shock protein levels (hsp70)	saprobic index	Peschke et al. 2014

Biomarkers: BAFA, Bacterial fatty acid; IDH, isocitrate dehydrogenase; LDH, lactate dehydrogenase; PUFA, polyunsaturated fatty acid. *Indices*: IBMWP, Iberian Biomonitoring Working Party index; IASPT, Iberian Average Score Per Taxon. n.i.: not identified at species level; n.d.: not determined.

Table 4.

Studies evaluating rivers and streams' ecological quality status based on biomarkers related to antioxidant defences and oxidative stress and structural indices using the benthic macroinvertebrate community (when determined).

Country	Macroinvertebrate species	Order (family)	Biomarkers	Structural indices	References
Antioxidant Defences					
Spain	*Hydropsyche exocellata*	-Trichoptera (Hydropsychidae)	SOD, CAT, $GSTP_X$	-IBMWP	Barata et al. 2005
Spain	*Hydropsyche exocellata*	-Trichoptera (Hydropsychidae)	CAT	-IBMWP, -IASPT	Damásio et al. 2011a
Spain	*Hydropsyche exocellata*	-Trichoptera (Hydropsychidae)	SOD, CAT	-ICM-Star (multimetric index), -iMMi-T (multimetric index)	Prat et al. 2013
Spain	*Hydropsyche exocellata*	-Trichoptera (Hydropsychidae)	SOD, CAT, GPx, GR, GSH, GSH/GSSG	-iMMi-T multimetric index	Puértolas et al. 2010
Spain	*Hydropsyche exocellata*	-Trichoptera (Hydropsychidae)	CAT	n.d.	De Castro-Català et al. 2015
Spain	*Dreissena polymorpha*	- (Dreissenidae)	SOD, CAT, GPx, GR, GSH, MT	n.d.	Faria et al. 2010a
Spain	*Dreissena polymorpha* *Corbicula flumínea* *Psilunio littoralis*	-Veneroida (Dreissenidae, Cyrenidae, Unionidae)	SOD, CAT, GPx, GR, GSH, MT		Faria et al. 2010b
Oxidative Damage					
Spain	*Hydropsyche exocellata*	-Trichoptera (Hydropsychidae)	LPO	-IBMWP	Barata et al. 2005
Spain	*Hydropsyche exocellata*	-Trichoptera (Hydropsychidae)	LPO	-IBMWP, -IASPT	Damásio et al. 2011a
Spain	*Hydropsyche exocellata*	-Trichoptera (Hydropsychidae)	LPO	-ICM-Star (multimetric index), -iMMi-T (multimetric index)	Prat et al. 2013
Spain	*Hydropsyche exocellata*	-Trichoptera (Hydropsychidae)	LPO, DNA strand breaks	-iMMi-T multimetric index	Puértolas et al. 2010
Spain	*Dreissena polymorpha*	-Veneroida (Dreissenidae)	LPO, DNA strand breaks	n.d.	Faria et al. 2010a
Spain	*Dreissena polymorpha* *Corbicula fluminea* *Psilunio littoralis*	-Veneroida (Dreissenidae, Cyrenidae, Unionidae)	LPO, DNA strand breaks		Faria et al. 2010b

Biomarkers: CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSH/GSSG, Glutathione redox status; $GSTP_X$, Selenium-independent glutathione peroxidase; LPO, lipid peroxidation; MT, metallothionein proteins; SOD, superoxide dismutase. *Indices*: IBMWP, Iberian Biomonitoring Working Party index; IASPT, Iberian Average Score Per Taxon. n.i.: not identified at species level; n.d.: not determined.

2.5.2.1. Neurotransmission

Organophosphorus (OP) and carbamate pesticides are known to inhibit type B esterases (i.e. a large group of serine hydrolases), including cholinesterases (ChEs) and carboxylesterases (CbEs), by binding to the active site and phosphorylating the enzyme (Barata et al. 2004; Van der Oost et al. 2005). Cholinesterases (ChEs) belong to the esterase family with the capacity of hydrolysing carboxylic esters (Eto 1974) and play an

important role in the maintenance of normal neural functions. ChEs inhibition has been used for decades in laboratory and *in situ* ecotoxicity studies as a biomarker of neurotoxicity to evaluate aquatic contamination (Damásio et al. 2011b; Domingues et al. 2010; Kristoff et al. 2010; Pestana et al. 2014). In vertebrates (e.g. fish), cholinesterase activity is well documented. ChEs are commonly divided into two broad classes: acetylcholinesterases (AChE) and butyrylcholinesterases (BChE) (Fulton and Key 2001), also known as pseudocholinesterases. AChE is responsible for hydrolysing the acetylcholine (present in vertebrates and invertebrates) into choline and acetic acid at cholinergic synapses and neuromuscular junctions (Kristoff et al. 2010), a process which is vital for the normal functioning of sensory and neuromuscular systems (Oliveira et al. 2012). Pseudocholinesterases are involved in the detoxification of several xenobiotics and can also prevent compounds from inhibiting AChE activity by binding to them and thus decreasing their free concentration in the organism (Almeida et al. 2010). AChE and BChE are primarily distinguished through their substrate specificity and they may also be distinguished by their sensitivity to selective inhibitors (Massoulie et al. 1993). AChE hydrolyses acetylcholine at a much higher rate than other choline esters and is inactive on butyrylcholine, and is specifically inhibited by anti-cholinesterase chemicals such as neurotoxic insecticides (organophosphorus and carbamate pesticides), which are designed to control invertebrate pests (Hassall 1990). Most of these compounds have low persistence in aquatic ecosystems, but the relative lack of species specificity has raised concerns about their potential to cause adverse effects on non-target wildlife populations, particularly invertebrates (Schulz and Liess 1999). After a reduction in exposure to insecticides, the recovery process of AChE activity depends on the type of insecticide, the species affected and the extent of AChE inhibition (Abdullah et al. 1994; Morgan et al. 1990). AChE can also be affected by non-specific inhibitors such as some metals, polycyclic aromatic hydrocarbons (PAHs) and even emerging pollutants such as pharmaceuticals, causing an over-accumulation of the neurotransmitter acetylcholine in the synaptic cleft or the neuromuscular junction, thus promoting prolonged electrical activity at nerve endings which may ultimately lead to death (Berra et al. 2006; Damásio et al. 2011a; Domingues et al. 2010; Garcia et al. 2000; Pestana et al. 2009, 2014; Santos et al. 2012; Schulz and Liess 2000; Siebel et al. 2010).

In field studies, the determination of AChE inhibition in benthic macroinvertebrate species has been successfully used as a biomarker for the presence of neurotoxic compounds (Berra et al. 2004; Damásio et al. 2011a; Maltby and Hill 2008; Minutoli et al. 2013; Xuereb et al. 2007; Table 2). For example, Minutoli et al. (2013) reported that the highest AChE inhibition in *Serratella ignita* larvae (Ephemeroptera) in the Alcantara River (Sicily, Italy) was observed in organisms collected from sites with intense

agricultural activity (Table 2). Another study conducted in the Titley Court stream (Herefordshire, UK) also reported a significant difference in AChE activity in gammarids (*Gammarus pulex*) during chlorpyrifos (organophosphorus pesticide) application (pesticide sprayed to stream edge). The same authors concluded that, although the short-term pesticide exposure caused effects in benthic macroinvertebrates at an individual level, these effects were apparently not translated to the population level, possibly because of a no-spray buffer zone (Maltby and Hill 2008; Table 2).

CbE hydrolysis a wide range of exogenous and endogenous esters and, in arthropods, B esterases such as CbEs are considered to be important detoxifying enzymes (Yuan and Chambers 1996). They have also been used as biomarkers of exposure to organophosphate and carbamate pesticides in benthic macroinvertebrates in field studies (Damásio et al. 2010, 2011a; Puértolas et al. 2010; Faria et al. 2010a; Table 2). They are believed to provide protection against OP and carbamate toxicity via two main mechanisms: 1) direct hydrolysis of ester bonds in OPs and carbamates and 2) as alternative stoichiometric phosphorylation/carbamylation sites, which could reduce the amount of pesticide available for AChE inhibition (Barata et al. 2004; Kristoff et al. 2010).

2.5.2.2. Energy metabolism

Lactate dehydrogenase (LDH) is a glycolytic enzyme involved in the anaerobic pathway of energy production. LDH catalyses the interconversion of pyruvate and lactate with concomitant interconversion of NADH to NAD+, assuming particular importance in muscular physiology under stress conditions, when high energy levels are required in a short period of time (De Coen et al. 2001; Diamantino et al. 2001). Increased LDH activity was previously observed in benthic macroinvertebrate species (caddisfly larvae) exposed to contamination in rivers (Damásio et al. 2011a; Prat et al. 2013; Table 3), as additional energy may be required to maintain standard levels of biochemical or physiological functions. Conversely, contaminants may prompt a decrease in LDH activity, for example, by binding to and inactivating the enzymatic molecule, or blocking its synthesis (Mishra and Shukla 1997).

The measurement of isocitrate dehydrogenase (IDH), a citric acid cycle enzyme, may provide valuable additional knowledge for understanding LDH response patterns, due to its role in the aerobic pathway of energy production and its contribution to antioxidant responses, where it is involved in the regulation of the cell redox balance (Jo et al. 2001; Lee et al. 2002). It has been successfully determined in invertebrate species

(Rodrigues et al. 2013), including benthic macroinvertebrates (Berra et al. 2004; Table 3).

2.5.2.3. Biotransformation enzymes

Biotransformation enzymes have long been used as biomarkers in various organisms, including freshwater benthic macroinvertebrates (Table 4). These enzymes act on xenobiotic metabolism through their conversion into more hydrophilic metabolites, and/or conjugation with important molecules, in order to facilitate their elimination (Van der Oost et al. 2003). Different environmental contaminants (e.g. oils, pesticides, PAHs) can alter these enzymes relative to control levels (Frasco and Guilhermino 2002; Kavitha and Rao 2009; Peña-Llopis et al. 2003; Vieira et al. 2009).

Xenobiotic biotransformation encompasses two major types of enzymes: phase I enzymes and phase II enzymes and cofactors. Phase I reactions are catalysed by the mixed-function oxidase (MFO) system (i.e. cytochrome P450 - cyt P450 -, cytochrome b5 - cyt b5 -, NADPH cytochrome; P450 reductase - P450 RED) which introduces (or modifies) a functional group (–OH, –COOH, –SH, –NO2) into the xenobiotic or its metabolites. The enzymes of phase II metabolism attach an endogenous ligand to this, often a more polar group (glutathione, sulphate, glucuronide, amino acid, etc.), leading to the formation of less hydrophobic compounds that are more easily excreted (Livingstone 1991; Van der Oost et al. 2003). An important enzymatic complex involved in the phase II biotransformation is the glutathione-S-transferase isoenzymes (GST). The key role of GST is to catalyse the conjugation of reactive electrophilic compounds with tripeptide glutathione (GSH) during phase II biotransformation, increasing their polarity and therefore enabling their excretion (Sáenz et al. 2010). GSTs play an important role in the cellular defence against oxidative damage and peroxidative products of DNA and lipids (Van der Oost et al. 2003). Due to the action of specific transporters, GSH conjugates are removed from the cell, thereby protecting crucial cellular proteins and nucleic acids from the action of reactive electrophilic compounds (Espinosa-Diez et al. 2015). In insects, some GST isoenzymes also display peroxidase activity (Ahmad 1992).

GSTs in insects have attracted attention due to their involvement in the defence against insecticides, mainly organochlorine (Lagadic et al. 1993) and organophosphate insecticides (Clark 1989; Hayaoka and Dauterman 1982). However, some laboratory studies have suggested that GSTs are not suitable for use as a biomarker of pesticide exposure in the species *Chironomus riparius* (e.g. Callaghan et al. 2001; Crane et al. 2002; Hirthe et al. 2001). Reports on laboratory studies have also found correlations

between high levels of GST and resistance to pyrethroids for several insect species (Grant and Matsumura 1989; Kostaropoulos et al. 2001; Lagadic et al. 1993; Reidy et al. 1990).

2.5.2.4. Antioxidant defences

Reactive oxygen species (ROS), i.e. oxygen free radicals and non-radical reactive species, are products resulting from the basic cellular metabolism of aerobic organisms. These reduction products of molecular oxygen (O_2) include the superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical ($OH^{\bullet}$). Moreover, many chemical pollutants such as pesticides, polychlorinated biphenyls (PCBs) (Orbea et al. 2002; Winston and Di Giulio 1991) and metals (Stohs and Bagchi 1995) cause oxidative stress by enabling the production of ROS, which can oxidise most cellular constituents such as proteins, lipids and DNA, and can significantly disturb vital cellular functions (Barata et al. 2005, Sahan et al. 2010). Transition metals (e.g. iron, copper, chromium and vanadium) are of particular interest because they simplify the conversion of $O_2^{\bullet -}$ into $OH^{\bullet}$ through the Fenton reaction. Other metals (e.g. cadmium, nickel, lead, aluminium, arsenic and mercury) are capable of depleting glutathione levels or displacing redox metal ions via metal induction, causing indirect oxidative stress (Stohs and Bagchi 1995).

All aerobic organisms have a suite of biochemical defence mechanisms, both of enzymatic and non-enzymatic origin, to prevent or reduce the formation of ROS (Choi et al. 2001; Mouneyrac et al. 2011). Non-enzymatic antioxidant defences consist of molecules of low molecular weight acting as free radical scavengers, such as glutathione (Kristoff et al 2008). The enzymatic defence mechanism includes superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase. The superoxide dismutase (SOD) is a group of metalloenzymes responsible for catalysing the dismutation of $O_2^{\bullet -}$ to O_2 and H_2O_2 (McCord and Fridovich 1969; Scandalios 1993). However, the H_2O_2 is still a harmful by-product and needs to be eliminated or degraded. H_2O_2 is subsequently detoxified by two types of enzymes: catalase (CAT) and glutathione peroxidase (GP_X), so activating a second enzymatic mechanism. Catalase (CAT) catalyzes the decomposition of H_2O_2 to H_2O (Aebi 1984), and glutathione peroxidase (GPx) catalyses the reduction of both H_2O_2 and organic hydroperoxides produced, for example by lipid peroxidation, using reduced glutathione (GSH) as a cofactor, which is oxidised to its oxidised form (GSSG). In animals, the principal peroxidase is a selenium (Se) - dependent tetrameric enzyme that catalyses the reduction of H_2O_2 to H_2O. The other

peroxidase reduces organic hydroperoxides to their corresponding alcohols, a process which is considered an important mechanism for protecting membranes from damage from lipid peroxidation (Stegeman et al. 1992).

Glutathione-S-transferase (GST) enzymes cannot reduce H_2O_2 but they can employ GSH in the reduction of a broad range of organic hydroperoxides. This peroxidase activity by GST is referred to as "selenium-independent peroxidase", although GST is not a true peroxidase (Stegeman et al. 1992). To maintain the reduction potential of the cell, the GSSG could be reduced to GSH again with the help of glutathione reductase (GR), with the concomitant oxidation of NADPH (from the Penthose Phosphate Pathway – PPP) to $NADP^+$ (Espinosa-Diez et al. 2015) or exported from the cell, like some GSH conjugates, via multidrug resistance associated proteins (Halliwell and Gutteridge 1999). Decreased GR activity may lead to GSH reduction if the reduction cannot be corrected by the synthesis of new glutathione molecules. This makes it possible to infer the most likely occurrence of oxidative stress by measuring significant increases in specific enzyme activities (e.g. SOD and CAT).

Although these antioxidant systems can demonstrate decreases, increases or both trends under stress conditions, this response is not contradictory as it depends on the intensity of exposure to the chemical substances (single or mixed contaminants, bioavailability) and the susceptibility of the exposed living species (Bocchetti et al. 2008; Faria et al. 2009; Regoli et al. 2002, 2003). In field surveys, significant increases of antioxidant enzyme activity have already been recorded in benthic macroinvertebrates collected from contaminated rivers (e.g. Barata et al. 2005; Damásio et al. 2011a; De Castro-Catalá et al. 2015; Faria et al. 2010a, b; Puértolas et al. 2010; Table 4). For example, De Castro-Catalá et al. (2015), studying the responses of *Hydropsyche exocellata* to priority and emerging pollutants in four Spanish rivers (Ebro, Llobregat, Júcar and Guadalquir) found a close relationship between CAT activity and the concentration of endocrine disrupting compounds (EDCs) and pharmaceutical active compounds (PhACs). The downstream sites (most contaminated) showed lower CAT activity and higher PhACs concentrations (Table 4).

GSH levels have a protective role against ROS by reacting with $O_2^{\bullet -}$, peroxy radicals ($ROO^{\bullet}$) and singlet oxygen ($^1O_2$), followed by the formation of GSSG and other disulfides (Meister 1988). GSH also acts as a reactant in conjugation with electrophilic substances. GSH homeostasis in the cell is not only regulated by its de novo synthesis, but also by other factors such as utilisation, recycling and cellular export. This redox cycle is known as the GSH cycle and incorporates other important antioxidant, redox-related enzymes (Espinosa-Diez et al. 2015). The ratio of reduced to oxidised glutathione (GSH/GSSG ratio), is possibly the main key factor in the non-enzymatic antioxidant

mechanisms of most living organisms (Haberhauer-Troyer et al. 2013). Normally, GSSG comprises only a small fraction of the total glutathione content. However, the production of ROS increases GSSG levels, thus reducing the GSH/GSSG ratio, indicating that GSH is being used as an antioxidant (Haberhauer-Troyer et al. 2013). The antioxidant enzymes GPx, and GST are in fact highly dependent on glutathione (GSH) to carry out their functions properly.

In a field study conducted in Saricay Creek (Çanakkale, Turkey), GSH levels increased significantly in *Asellus aquaticus* (Isopod) from the most polluted sites, with higher levels of metals and other environmental factors compared to the clean sites (Kaya et al. 2014). GSH levels can be increased due to an adaptive reaction to slight oxidative stress, through an increase in its synthesis. However, a severe oxidative stress may suppress GSH levels due to the loss of adaptive mechanisms and the oxidation of GSH to GSSG. The conjugation of pollutants or their secondary products with GSH directly or by means of GSTs, decrease GSH levels. If generation of GSSG is higher than the reduction by GR back to GSH, then GSSG accumulates and is translocated outside the cell by specific transporters to avoid NADPH exhaustion (Wu et al. 2011). For example, Faria et al. (2010a) observed lower levels of GSH as well as higher activities of antioxidant enzymes (e.g. SOD, CAT, GST) and higher levels of biomarkers of oxidative damage (LPO, DNA strand breaks) in zebra mussels (*Dreissena polymorpha*) collected from a Spanish river (the Ebro River) at the site of and downstream from a chlorine-alkali plant with high levels of mercury, compared to the upstream sites (Table 4). Such signs of oxidative stress were also related to the bioaccumulation of organochlorides (e.g. polychlorinated biphenyls - PCBs -, hexachlorobenzene - HCB -, and dichlorodiphenyltrichloroethane - DDT) and metals (e.g. mercury and cadmium) (Faria et al. 2010a).

2.5.2.5. Oxidative stress: markers of oxidative tissue damage

An impaired antioxidant defence mechanism significantly increases the organisms' sensitivity to oxidative stress, resulting in damage to cellular lipids, proteins and DNA (Cossu et al. 2000). Lipid peroxidation (LPO) is a process that results from the destruction of membrane lipids due to free radical reactions (Oliveira et al. 2009) and is one of the most studied effects indicative of oxidative stress. Degradation of membrane lipids by free radical attack results in the production of compounds such as malondialdehyde (MDA). The presence of MDA is indicative of oxidative damage and the reaction of MDA with 2-thiobarbituric acid (TBA) is one of the most widely used

estimators of oxidative stress (Oakes and Van Der Kraak 2003). DNA modifications, such as single and double strand breaks, can be produced either directly by the toxic chemical (or its metabolite) or by the processing of structural damage (Shugart et al. 1992; Viarengo et al. 2007). When the DNA strand breaks it can be repaired incorrectly or not at all. This can have adverse effects on the organism's state of health (Lam 2009). The evaluation of lipid peroxidation using thiobarbituric acid reactive substances (TBARS) and the amount of DNA strand breaks have been used as biomarkers in benthic macroinvertebrates for monitoring particular contaminated ecosystems (e.g. Damásio et al. 2011a; Faria et al. 2010a, b; Prat et al. 2013; Puértolas et al. 2010; Table 4).

Other biomarkers involved in different physiological processes have seldom been used, although also showing some potential to provide useful information, namely *p*-nitrophenylacetate esterase (pNPAE), α-naphtylacetate esterase (NAE), alkaline phosphatase (AP), L-alanine (Bonzini et al. 2008), benzo(a)pyrene monooxygenase (BPMO) (Minutoli et al. 2013), glucose-6-phosphate dehydrogenase (G6PDH), malic enzyme (ME) and alcohol dehydrogenase (ADH) (Berra et al. 2004).

2.5.3. Combined biomarkers and community-based field evaluations

Community indices are able to assess combined effects of environmental factors and chemical pollution that can affect the natural communities (e.g. acidification, flow modification, organic pollution, habitat degradation) (Bonada et al. 2006). However, they are not reliable indicators of ecological impairment caused by specific contaminants (Baird and Burton 2001). The responses of these indices are not specific, and frequently integrate the impact of the mixtures of contaminants, of abiotic stress factors, and individual and taxa-specific sensitivities, making it more difficult to establish cause and effect relationships. More importantly, they can only detect sizeable effects that often consist of the elimination of one or several species from a particular site (Damásio et al. 2011a). They are therefore not suitable for diagnosing subtle biological impairments caused by physiological effects resulting from chronic exposure to low levels of contamination. If the focus of contaminant studies in rivers or streams is only based at the community level, chronic or subtle biological effects that might be taking place in apparently healthy ecosystems but would not be detected in time to be reversed (Maher et al. 1999). In fact, before structural changes occur in aquatic systems, pollutants first act at a subcellular level, causing individual alterations to important vital functions (e.g.

feeding, metabolism, mobility or reproduction). Biomarker batteries have become an increasingly useful tool for modern environmental assessment as they can help to early diagnose and characterise impaired health status resulting from overall exposure to chemicals and other environmental stressors.

It is now widely recognised that the WFD requires new ecological perspectives based on multidisciplinary and holistic approaches through the integration of multiple lines of evidence. Biomarkers and community indices appear to be complementary approaches that enable an overall insight into the quality of the water body (e.g. Allan et al. 2006; Hagger et al. 2006; Martinéz-Haro et al. 2015). Furthermore, while chemical analyses provide data on the presence and/or concentrations of individual chemicals in the studied system, which may not always be related with a toxic effect (Martinéz-Haro et al. 2015), the biomarker approach is able to provide information on the early exposure to and/or effects of the chemicals (including the combined effects of mixtures of compounds, both known and unknown) on aquatic organisms, rather than a mere quantification of their environmental levels. Chemicals exist in different forms and may interact in organisms in different ways (e.g. additively, antagonistically or synergistically), altering their toxicity relative to that of single compounds (Lam 2009). Biomarker measurements integrate effects resulting from different exposure routes (water, food, sediment) over time and geographically over the spatial range of the sentinel species, e.g. the limited migration patterns or sessile behaviour of benthic macroinvertebrates may aid in the identification of “hot spots” of contamination. Biomarkers can also provide evidence of exposure to compounds that do not bioaccumulate or are rapidly metabolised and eliminated.

In support of the upcoming WFD revision in 2019, the research project SOLUTIONS and the European monitoring network NORMAN have analysed the challenges facing the practical implementation of the WFD with regard to chemical pollution, suggesting possible monitoring improvements (Brack et al. 2017). According to this analysis, the list of PSs and CECs that must be monitored in European surface waters (EU 2013, 2015) is far from comprehensive in terms of the numerous compounds and their metabolites that enter aquatic systems and mixtures of these compounds that may cause severe effects on aquatic organisms and human health. Connections must be established between external levels of exposure, internal levels of tissue contamination and early adverse effects. This problem requires a novel and more holistic approach to address chemical pollution in the environment as a whole. The use of effect-based tools such as biomarkers and trigger values to address priority mixtures of contaminants in monitoring schemes will help to bridge the gap between chemical contamination and ecological status, and will provide indications on toxic chemicals as a

probable cause of deterioration (Brack et al. 2017). The use of biomarkers as a supplementary approach can therefore help reduce chemical monitoring efforts and increase confidence in the risk assessment of water bodies, delivering potentially long-term efficiency savings. These can be further maximised if the set of biomarkers employed within the risk assessments are cost-effective, easy to use and fast (Hagger et al. 2008). However, where effect-based trigger values are exceeded, solution-focused procedures are required to identify the drivers of the measured effects, in order to initiate effective management activities before and along with risk assessment (Brack et al. 2017).

Most studies combining biomarkers and community-based approaches for the assessment of the ecological quality of rivers have been conducted in Spain (Catalonia), in some heavily industrialised and urbanised river basins (e.g. Barata et al. 2005; Damásio et al. 2011a; Prat et al. 2013; Puértolas et al. 2010; Tables 2, 3, 4). A tolerant caddisfly larvae species (*Hydropsyche exocellata*) was used to detect environmental fluctuations produced by different sources. In general, these studies have shown that the use of a battery of sensitive biomarkers, comprising a large set of biochemical responses, improves the success in determining the causes of deterioration in a water body and shows whether pollutants are the main reason for not achieving a “good status”. Barata et al. (2005) found that, compared to the clean upstream sites of the Llobregat River system (NE, Spain), salt in the water and increasing levels of metal body burdens in *H. exocellata* larvae collected from the most polluted sites (downstream sites close to industrial and urban areas), were related to the activity of antioxidant (CAT, GST) and the levels of oxidative stress (LPO) biomarkers. Site differences in *H. exocellata* biomarker responses were also observed in a later work conducted in similar sampling locations of the Llobregat River (Puértolas et al. 2010). These included significantly increased activities of GST and GPx, a strong reduction in the redox status and higher levels of LPO three days after glyphosate herbicide was sprayed on the river banks. Activities of SOD and CAT, levels of GSH and the amount of DNA strand breaks were also determined, but these did not change significantly before and after herbicide exposure (Puértolas et al. 2010). Another study, carried out by Prat et al. (2013), aimed at evaluating the quality of the Llobregat River after the introduction of reclaimed water from a waste water treatment plant. Several structural metrics were determined to evaluate the biological quality. Although the ecological status remained “poor”, structural metrics indicated slightly lower values after the introduction of the treated water. In line with this, significant toxic effects were observed in *H. exocellata* larvae using biomarkers. A decrease in the antioxidant and detoxification defences (lower activities of CAT and GST, respectively), and consequent increased levels of lipid peroxidation suggest a

decline in the river's ecological status. According to the authors, if the concentration of some pollutants (mainly salts, residual chloride and ammonia) would persist in the reclaimed water, *H. exocellata* would disappear and the river's condition would deteriorate to a "bad" ecological status. An evaluation of Besós River (Besós River Basin; NE, Spain) was also carried out (Damásio et al. 2011a). By studying the biochemical responses of *H. exocellata* to general degradation in the Besós River, the authors reported that biomarkers varied significantly throughout the sampling sites: they were able to distinguish samples from sites classified with good (upstream sites) and deteriorated (downstream sites) ecological status. Biotransformation (GST), antioxidant (CAT), metabolic (LDH) enzymes and biomarkers of oxidative damage (LPO, DNA strand breaks) increased from upper to downstream locations. The macroinvertebrate community was affected mainly by salinity, although antioxidant and metabolic enzyme activities were possibly associated with the presence of detergents, organochloride pesticides and PAHs. In addition to biological metrics, biomarkers therefore provided additional information when diagnosing the effects of different environmental factors threatening macroinvertebrate communities.

Although biomarkers are extensively used in ecological risk assessment of aquatic ecosystems, only a few studies employ combined biomarker and macroinvertebrate community approaches. Also, with few exceptions studies on freshwater benthic macroinvertebrates are generally based on a single and tolerant species (Kaya et al. 2014; Minutoli et al. 2013; Olsen et al. 2001). Berra et al. (2004) determined enzymatic biomarkers linked to neurotransmission, energy metabolism, biotransformation and antioxidant defences in different macroinvertebrate families, either tolerant or sensitive to contamination (insect families of the orders Diptera, Ephemeroptera, Odonata, Plecoptera and Trichoptera, and crustaceans and oligochaetes of the Gammaridae and Lumbricidae families, respectively) in two Italian rivers (the Taro and Ticino). This study showed that biomarker responses differed significantly among families (Berra et al. 2004). Bonzini et al. (2008), studying the stress responses of four taxonomic groups (Gammaridae, Asellidae, Hirudinea and Oligochaeta) from two other Italian rivers (the Meolo and Livenza) under anthropogenic pressure (pesticides and other organic contaminants) using enzymatic biomarkers, also reported that different taxonomic groups have different sensitivity to environmental stressors (Bonzini et al. 2008). Such findings suggest that the evaluation of a single species may result in either under or over estimation of the risk, depending on the taxa selected. Similar to a multi-biomarker approach, multi-taxa approaches are expected to provide a more integrative and complementary view of ecosystem health by encompassing diverse forms of biological integration of the environment, multiple

exposure routes and different species' sensitivities, and allowing for the validation of results from biomarkers' and species' evaluations (Duarte et al. 2017).

The difficulty or impossibility of identifying benthic macroinvertebrates at the species-level have contributed to limiting biomarker use in environmental risk assessments using these organisms. The identification of benthic macroinvertebrates at species-level is extremely laborious and time-consuming and therefore expensive (Marshall et al. 2006). Moreover, frequent identification errors occur at species-level (due to the necessity of extensive taxonomic expertise) and several freshwater taxa simply lack morphological diagnostic characters at the larval and even the adult stages ("cryptic species": Cook et al. 2008; Liu et al. 2003; Weiss et al. 2014). For example, immature stages of chironomids are commonly the most species-diverse and abundant macroinvertebrates in freshwater ecosystems. However, they are not routinely identified to species-level in bioassessments because they are poorly described or their identification to species or even genus level is technically difficult or impossible using traditional morphology-based methods (Jones 2008).

If it is assumed that the determination of biomarkers in organisms identified to higher taxonomic levels than species may include species with different sensitivities, previous studies suggest that the biomarker measurements at higher taxonomic levels than species may be effective in detecting subtle gradients of toxic substances and their effects on the exposed biota (Berra et al. 2004; Bonzini et al. 2008). Since biomarker responses have a phylogenetic component, sister species (e.g. species in the same genus) are likely to show similar physiological responses (Colin et al. 2016). This would indicate that further studies including different biomarkers, environmental stressors, macroinvertebrate taxa and river types are necessary to establish efficient and cost-effective biomarker strategies indicative of future ecological damage.

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03

CHAPTER

Assessing the ecological status of small Mediterranean rivers: benthic macroinvertebrates and macrophytes as complementary indicators

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Journal-Article

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Assessing the ecological status of small Mediterranean rivers: benthic macroinvertebrates and macrophytes as complementary indicators

3.1 Abstract

Rivers are amongst the most threatened ecosystems in Europe. To prevent further degradation and to improve their ecological status, effective mitigation and restoration actions are needed. According to the Water Framework Directive (WFD; Directive 2000/60/EC), those actions are primarily based on the precision of the ecological assessment results. The present study was conducted between July 2013 and September 2014 and aimed to assess the ecological status of two small Mediterranean rivers through the analysis of benthic macroinvertebrates (North Invertebrate Portuguese Index, $IPtI_N$) and macrophytes (Macrophyte Biological Index for Rivers, IBMR; Riparian Vegetation Index, RVI). Specific objectives were to compare the performance of the two biological quality elements (BQEs) and the usefulness of their information for river management, and to confirm adequate temporal windows to develop the monitoring surveys. Physico-chemical and hydromorphological quality elements were also monitored to support the interpretation of the BQEs assessed.

High levels of nutrients were detected in some sites of the Âncora River and in all sites of the Ferreira River, particularly during the spring and summer. Both rivers were found altered mainly, in terms of floristic composition of the riparian communities, with the riparian forest dominated by several exotic woody species and the forbs fringe dominated by nitrophylous communities. The RVI index revealed a low riparian cover in the Ferreira River sites and a high cover and number of alien species (mostly invasive) in both rivers. The evaluation of ecological quality obtained with the $IPtI_N$ was lower than that obtained with the IBMR for both rivers. Therefore, the evaluation of ecological status of rivers using only the macrophytes' responses to nutrient enrichment (IBMR) provided a partial evaluation of the effects of the stressors affecting the integrity of the river ecosystems. The RVI contributed to a better evaluation of the ecological status of the rivers and provided also support for planning decisions regarding the management of both systems.

The evaluation of the ecological status of small fluvial systems benefits therefore from an integrated multidisciplinary approach allowing a more accurate diagnosis of their ecological status. Mitigation of diffuse pollution and restoration of the riparian zones are a priority to improve the ecological status of the studied rivers, both located in sites of European interest.

Keywords: Macroinvertebrates; Macrophytes; Ecological status; Water Framework Directive; Conservation value

3.2. Introduction

Although awareness about its vital importance has been continuously increasing, the biodiversity of Mediterranean rivers is still severely threatened. Hydromorphological changes, along with increased chemical pollution, nutrient loadings and extreme weather events, such as droughts and floods, heavily influence the rivers' ecological integrity and aquatic biodiversity (Bonada and Resh 2013; Hershkovitz and Gasith 2013). To prevent further deterioration and to improve the waterbodies' ecological quality, the European Water Framework Directive (WFD; Directive 2000/60/EC; EC 2000) established that all EU member states should protect, enhance and restore the aquatic environment through the implementation of measures aimed at maintaining or achieving a "good status". For surface waters, the "good status" (i.e. both "good" ecological and chemical statuses) should be attained by 2027 at the latest.

The ecological status is defined as an expression of the quality of the structure and functioning of aquatic ecosystems associated with surface waters and it is assessed based on biological quality elements (BQEs), together with the evaluation of hydromorphological and physico-chemical quality elements (EC 2000). The use of complementary BQEs, as established by the WFD, is based on the premise that multiple BQEs can better detect the effects of multiple stressors (Hering et al. 2006b). However, responses to human induced disturbances have been mainly analysed with an individual BQE and only few authors compared the sensitivity of the four BQEs recommended by the WFD for river monitoring (fish, phytobenthos, macrophytes and benthic macroinvertebrates) (e.g. Johnson et al. 2006b; Hughes et al. 2009; Marzin et al. 2012; Lainé et al. 2014). These studies showed that response patterns and robustness differed considerably among BQEs, stressors and river types (Hering et al. 2006a;

Johnson et al. 2006a, b; Hughes et al. 2009; Johnson and Hering 2009; Marzin et al. 2012; Turunen et al. 2016). In general, all BQEs seemed to be more responsive to water quality (eutrophication/organic pollution) than to hydromorphological degradation (e.g. Hering et al. 2006a; Johnson et al. 2006b; Marzin et al. 2012). Nevertheless, since BQEs responses are often correlated, it has been recognised that it is not necessary to monitor all BQEs (Hering et al. 2006b). Ideally, the selection of the BQEs should be based on their greatest sensitivity to the stressor as well as on uncertainty associated with the respective metrics (Johnson et al. 2006a; Marzin et al. 2012). The choice of suitable BQEs/metrics is important in a limited timeframe and budget context, when authorities look for a reduction in the environmental monitoring costs, while maintaining a good assessment of the status of their waterbodies.

Indices based on these four BQEs were harmonised during the intercalibration exercise (IC), developed to ensure the comparability across EU countries of the classification results obtained. Geographical intercalibration groups (GIGs), aggregating countries or parts of countries sharing common intercalibration types, were created for the IC purpose (EC 2011). The Mediterranean Geographic Intercalibration Group (MedGIG) is one of these groups and includes regions/countries from the Mediterranean basin and with a Mediterranean climate (including Portugal, Spain, France, Italy, Slovenia, Greece and Cyprus) (Ferreira and Sabater 2014). This climate is characterised by sudden heavy rainy episodes that generally take place during the autumn and spring months, with driest conditions prevailing during summer (Lake 2003; Morais et al. 2004; Pardo and Álvarez 2006). During the first phase of the IC (2003-2007), in the MedGIG, it was only possible to intercalibrate the BQEs benthic macroinvertebrates and phytobenthos (EC 2008). The second phase of the IC (2007–2011) not only allowed the determination and promotion of the precision and confidence level of the classification results of the first phase, but also enabled the intercalibration of the remaining BQEs in rivers, namely fish and macrophytes (Aguiar et al. 2014b; Almeida et al. 2014; Feio et al. 2014a, b; Segurado et al. 2014). Macroinvertebrates are commonly used for assessing the effects of multiple stressors such as organic pollution, hydromorphological degradation, acidification and sedimentation (e.g. Larsen et al. 2010; Lorenz et al. 2004; Sandin et al. 2004). Macroinvertebrates have short generation times, ranging from weeks to months and may respond more rapidly to environmental changes than organisms with relatively longer generation times, being considered as both early- and late- warning indicators (Hering et al. 2006b). There is also evidence of stable responses of plant diversity and abundance to abiotic factors, and especially to nutrient enrichment, sedimentation and hydrological alterations (Aguiar et al. 2014b). Furthermore, plant communities have the capacity to incorporate the effects of successive anthropic

disturbances over long periods of time, from months to years, being good indicators for the monitoring of long-term changes, as late-warning indicators (Aguiar et al. 2014b; Hering et al. 2006b). However, there is still a need for comparative information about their sensitivity and adequacy for environmental monitoring of small rivers' quality status.

The present study thus aimed at evaluating the usefulness of benthic macroinvertebrates and macrophytes in assessing the ecological status of two small Mediterranean rivers, comparing the performance of both indicators. An integrated chemical-biological effects approach was used to answer the following questions: i) are the studied rivers similar in terms of physico-chemical, hydromorphological and biological parameters?; ii) which is the ecological status of these rivers, as indicated by the recently revised/intercalibrated macroinvertebrate and macrophyte indices, as well as by the macrophyte-based index of biotic integrity Riparian Vegetation Index?; iii) does temporal data support late spring as the most reliable for evaluation of the ecological status of the small Mediterranean rivers investigated?, iv) is the quality status information provided by macroinvertebrates and macrophytes similar or complementary?

3.3. Materials and methods

3.3.1. Study area and sampling sites

Two Northern-Portuguese rivers were studied (Fig 3A). The Âncora River (AR; Fig. 3A, B) springs from Serra de Arga, in the Viana do Castelo municipality (spring altitude: 816 m) and runs for approximately 17.91 km through a steep bedrock, before flowing directly into the Atlantic Ocean, in the Caminha municipality. The Ferreira River (FR; Fig. 3A, C) springs in Paços de Ferreira municipality (spring altitude: 550 m), has an approximate length of 22.30 km and joins the River Sousa in Gondomar municipality (Monteiro et al. 2005). Within the WFD implementation procedure, both the AR and FR were classified as "small sized streams" (catchment area: < 100 km^2), which reflects the country's northern climate with high annual average precipitation (mean: 1190.25 mm ± 357.80) and low annual average temperature (mean: 12.42 °C ± 1.26) (INAG 2008a).

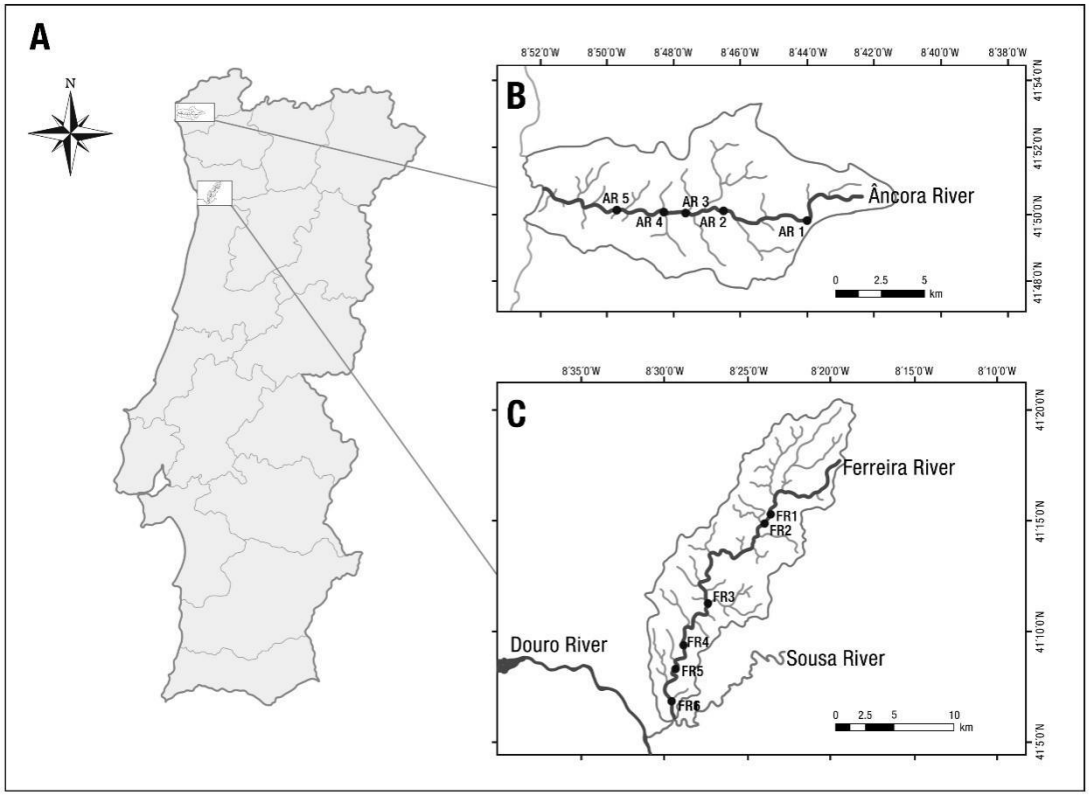

Fig. 3. Geographical situation. (A) Location of the hydrographic basins of River Âncora, B, and River Ferreira, C (rectangles), in Portugal mainland; (B) Details of the hydrographic basin of the Âncora River and the distribution of the sampling sites in the River Âncora (AR1 to AR5); (C) Details of the hydrographic basin of the Ferreira River and the distribution of the sampling sites in the River Ferreira (FR1 to FR6).

Both rivers flow in siliceous rocks (schist, granite), presenting low mineralization (INAG 2008a). In the hydrographic basin of the AR, the main sector of activity is the primary sector (PGRH1 2012). The hydrographic basin of the FR is dominated by significant urban and industrial activity, followed by agriculture (Monteiro et al. 2005).

Water, biological and sediment sampling took place between July 2013 and September 2014, at 5 sampling sites in the AR (AR1 to AR5, upstream to downstream; Fig. 3B) and at 6 sites in the FR (FR1 to FR6, upstream to downstream; Fig. 3C). Sampling sites (100 m long sections of the river channel on each site) were selected based on recommendations of national authorities, e.g., representing different types of meso-habitats in terms of substrate, shading, depth, stream velocity and water movements (INAG 2008b, c). AR sites are all integrated in the Natura 2000 Network (PTCON0039, Site “Serra de Arga”). Only one of the FR sampling sites (FR4; Fig. 3C) is also included in the Natura 2000 Network (PTCON0024, Site “Valongo”), but surrounded by areas with high risk of forest fires, urban pressure, sources of organic pollution, intensive forestry and recreational and leisure activities (Monteiro et al. 2005; PGRH3 2016).

3.3.2. Physico-chemical quality elements

Water physico-chemical parameters were monthly monitored. Water temperature (°C), pH, dissolved oxygen concentration (mg O_2/L) and percent saturation (% DO), total dissolved solids (mg/L), conductivity (µS/cm) and salinity (PSU) were measured *in situ* using a field probe (portable Multiparameter Meter*;* HI 9829*,* Hanna Instruments). Water samples were collected (at ± 5 cm from the surface) in 500 mL polyethylene bottles and transported to the laboratory where chemical oxygen demand (mg/L), nitrates (mg/L), nitrites (mg/L), ammonium ion (mg/L), total phosphorus (mg/L) and water apparent colour and true colour (PCU) were determined using a multiparameter bench photometer (C 99&200) following the manufacturer´s protocols (Hanna Instruments, 2014). Total suspended solids (mg/L) were analysed according to APHA (1992). Water physico-chemical parameters were classified considering the maximum limits for the "good" ecological status in Northern Portuguese rivers (INAG 2009).

3.3.3. Hydromorphological quality elements and sediment analysis

Hydromorphological parameters, organic matter content in sediments and sediment granulometry were seasonally monitored (summer and autumn 2013 and spring and summer 2014), except in winter due to high river discharge during this season. The characterisation of the rivers´ channel and riparian corridor was based on hydromorphological and structural variables, respectively, and carried out using the River Habitat Survey methodology (RHS; Raven et al. 1997, 2002, 2009). Hydromorphological river quality is expressed through the Habitat Quality Assessment (HQA) and Habitat Modification Score (HMS) indices, calculated from RHS survey information. HQA provides a broad indication of the overall habitat diversity in the channel and in the riparian corridor for the biological communities. Higher HQA scores represent more diverse sites. HMS provides an indication of artificial modifications in the river channel morphology (Raven et al. 2009). The field inventory was carried out according to the manual for the application of the RHS (EA 2003) in a 500 m segment along the river, at each sampling site. The data obtained in the field surveys was introduced and analysed using STAR RHS software (version 1.2), allowing an automatic calculation of the HQA and HMS indices. In Portugal, limit values for both indices were only defined for the class I, i.e., high ecological quality, thus separating scores

corresponding to this class, from lower-quality scores. The Habitat Modification Score (HMS) falls into one of five modification categories defined (ranging from 1 = near-natural to 5 = severely modified).

Other indices were determined in order to obtain a better hydromorphological characterization of both rivers and to achieve a more solid integration of the results obtained with the RHS methodology. The Riparian Forest Quality index (or *Qualitat del bosque de ribera,* QBR; Munné et al. 2003) is a simple method to evaluate riparian habitat quality, based on four components of riparian habitat: total riparian vegetation cover, cover structure, cover quality and channel alterations. It also takes into account differences in the geomorphology of the riparian vegetation from its headwaters to the lower reaches. The QBR index is obtained by the sum of the four scores of the four components of riparian quality, rating riparian quality according to 5 classes (I – high to V – bad), with a total score ranging from 0 (extreme degradation, bad quality) to 100 (riparian habitat in natural condition). The Channel Quality Degree index (or *Grau de Qualidade do Canal*, GQC; Cortes et al. 1999) allows evaluating the morphological condition of a lotic section through the analysis of 8 variables (e.g. presence of retention structures, artificial alteration of the river banks, channel heterogeneity). Each variable presents four levels of degradation, with the minimum score corresponding to a greater impact. The index value is obtained by the sum of the scores given to each variable and ranges from ≤ 13 (completely changed channel) to ≥ 31 (unchanged channel, natural state); its final value falls into one of the 5 classes of channel quality (I – high to V – bad).

Hydrological parameters were measured at 10 equidistant channel cross-sections (at 3 equidistant points in each cross-section) at each sampling site. The measured parameters were: average width (m; ultrasonic laser pointer), average depth (m; extendable graduated measuring), average flow velocity (m/s; speedometer) and average river discharge (m^3/s; Platts et al. 1983).

Sediment samples (4 replicates at ± 1 cm depth) were collected at each sampling site to determine sediment organic matter (OM) content and granulometry. OM content was determined by combustion (550 °C for 3 h) of previously dried samples (60 °C, for 24 h). It was calculated as percent weight loss on ignition of oven dried material and it is approximately equivalent to organic matter content (Strickland and Parsons 1972). The sediment used for particles size distribution analysis was previously dried in an oven (± 12 h). Grain size analysis was carried out by mechanical separation through a column of sieves with different mesh sizes that correspond to the different classes of sediment on the Wentworth scale (2 mm < granule + pebble < 64 mm; 1 mm < very coarse sand < 2 mm; 0.5 mm < coarse sand < 1 mm; 0.0622 mm < very fine + fine + medium sand < 0.5

mm; silt + clay < 0.0622 mm). After separation, each fraction was weighed and expressed as a percentage of the total weight (Wentworth 1922).

3.3.4. Biological quality elements

Benthic macroinvertebrates and macrophytes were seasonally monitored (summer and autumn of 2013 and spring and summer of 2014) and sampling followed national guidelines for the WFD implementation (INAG 2008b, c).

Samples were analysed in laboratory, where organisms were sorted out, counted and identified up to the genus level (except for Oligochaeta – to class level – and Chironomidae – to subfamily level) (Tachet et al. 2002). The ecological quality was assessed applying the North Invertebrate Portuguese Index ($IPtI_N$; INAG 2009; EC 2013). The final quality value recorded was expressed as the Ecological Quality Ratio (EQR), which is obtained dividing the $IPtI_N$ value by the reference value for the specific river type, and a quality class was assigned (I – excellent to V – bad). To improve our understanding of the environmental pressures acting upon the studied rivers, including organic contamination, the total number of individuals ($Log_{10}N$, being N the total No of individuals) and measures of richness (No families), composition (percent of Ephemeroptera, Plecoptera and Trichoptera orders, % EPT; % Hydropsychidae; % Chironomidae) and diversity (Pielou's evenness index, E; Shannon-Wiener diversity index, H'), as well as biotic indices (Biotic Belgian Index, BBI, De Pauw and Vanhooren 1983; Iberian Biological Monitoring Working Party Index, IBMWP, Alba-Tercedor and Sánchez-Ortega 1988) were determined.

All macrophyte species of the channel and banks were recorded along a 100 m stretch of the river (with a minimum sampling area of 2500 m^2), up to 50 m upstream of the macroinvertebrate sampling sites. Terrestrial species were also surveyed. Surveys were made by wading upstream in a zig-zag manner across the channel or by walking on banks. Then a downstream re-wade in the river stretch was done to ensure that all species were recorded, and to confirm the percentage cover of each species recorded in the first assessment. Specimens of bryophytes were surveyed in all existing microhabitats within the study reach (e.g. woody debris, rocks), in the channel, on banks, and on trees up to 0.5 m above ground level (Aguiar et al. 2014b; INAG 2008c). Percentage cover of each species was estimated by two experts and then compared to minimize estimation errors. Vascular plants and bryophytes were identified at the species-level (Aguiar et al. 2014b; INAG 2008c); the nomenclature followed *Flora Europaea* for vascular plants, Paton (1999) for liverworts and hornworts, and Smith

(2006) for mosses. The assessment of the ecological quality of both rivers was done using the Macrophyte Biological Index for Rivers (IBMR; Aguiar et al. 2014a; EC 2013; Haury et al. 2006), which is based on the occurrence and abundance, in water and on contact areas therewith, of indicator species, i.e., species that are sensitive to pollution, especially nutrients. The final value was expressed as EQR and a class of quality was assigned (I – high to V – bad).

The Riparian Vegetation Index (RVI; Aguiar et al. 2009) was determined for the global bioassessment of river quality, using the response of the vegetation to an array of disturbances. RVI is a multimetric index, based on compositional metrics (e.g. proportion of alien and endemic species) and functional metrics associated with life cycle and reproduction (e.g. proportion of perennial species), and with trophic status (e.g. proportion of nitrophylous species) (Aguiar et al. 2011b). Scoring metrics are estimated or evaluated using cover, proportion or number of species in functional groups (e.g. aliens, nitrophylous, ruderals) and species attributes (e.g. life form, reproduction strategies), and indicator taxa (e.g. *Carex elata* spp. *reuteriana*). The RVI for a site was obtained by the sum of the quality scores of all the metrics, less the total number of metrics for the Portugal mainland region (North region: 10 metrics). Each metric score ranged from five points for sites close to a reference condition to one point for a high level of human disturbances, totalling 0 to 40 for the North region (Aguiar et al. 2009). The final value was expressed as EQR, which is obtained dividing the RVI value by the reference value for the specific river type, and a class of riparian vegetation quality, in terms of structure and function, was assigned (I – high to V – bad).

3.3.5. Statistical data analysis

Spatial and seasonal variations of physico-chemical, hydromorphological and biological parameters were analysed using the non-parametric Kruskall-Wallis test, since most variables failed the Shapiro-Wilk normality test and/or the Levene test for homogeneity of variance across groups. When significant differences were found, the post-hoc pairwise Wilcoxon rank sum tests, corrected for multiple testing, was applied to identify significant differences between groups (Hollander and Wolfe 1973). For all tests a significance level of 0.05 was considered.

Multivariate analyses were applied to identify possible spatial and seasonal patterns in the multivariate space. A detrended correspondence analysis (DCA) was used to calculate the relative length of gradient to decide on the appropriate data analysis technique (i.e. use of unimodal or linear response analyses). Since all of our DCA

gradients lengths were < 2, samples were further submitted to a correlation-based Principal Component Analysis (PCA, centred and standardized to unit variance) for a multivariate assessment of water physico-chemical parameters, hydromorphological indices and sediment parameters (percent of organic matter and granulometry), total number of macroinvertebrates individuals and metrics of richness, composition, biotic and multimetric indices based on the benthic macroinvertebrate community. Because the macrophyte index IBMWP was only determined once per sampling site and season, and because index scores were very similar, this index was not included in the multivariate analyses. Given the very strong correlations found between conductivity and total dissolved solids (Spearman rho ≥ 0.988), conductivity and salinity (Spearman rho ≥ 0.999), and dissolved oxygen concentration (DO) and percent saturation (% DO) (Spearman rho = 0.919), only conductivity and dissolved oxygen were considered in the multivariate analysis.

The spatial variation of the macroinvertebrate-based North Invertebrate Portuguese Index ($IPtI_N$) and the macrophyte-based Macrophyte Biological Index for Rivers (IBMR) and Riparian Vegetation Index (RVI) was visualized in Tukey Boxplots, showing data distribution, medians and quartiles. The boxplot´s rectangle shows the interquartile range (IQR) from the first quartile (25th percentile) to the third quartile (75th percentile) of the distribution; the whiskers range from the minimum value to the maximum value.

Box-plots and statistical tests were done using the Statistical program R, version 3.4.2 (R Core Team 2017). DCA and PCA were performed and plotted using CANOCO 4.5 (Ter Braak and Smilauer 1998).

3.4. Results

3.4.1. Physico-chemical quality elements

Water physico-chemical parameters showed both seasonal and spatial variations in the Âncora (AR) and Ferreira (FR) rivers. Significant differences between rivers, were found for almost all of the physico-chemical parameters analysed (temperature, $p = 0.0092$; pH, conductivity, salinity, total dissolved solids, total suspended solids, apparent colour, true colour, nitrates, nitrites, ammonium ion and phosphorus, $p < 0.0001$), with higher values occurring in the FR. Significant differences among seasons were found for water

temperature, dissolved oxygen concentration (DO) and percent of saturation (% DO), conductivity, total dissolved solids (TDS), phosphorus, nitrates and nitrites in both rivers, and salinity, pH and ammonium ion in the FR only (Table 5). Significant differences among sites were found for conductivity and salinity in both rivers and for TDS, total suspended solids (TSS), apparent colour, and all nutrients in the FR only (Table 5).

AR and FR presented good oxygenation, with higher DO means occurring in winter (Table 5). On the contrary, conductivity and TDS, showed lower means in winter compared to the remaining other seasons, in both rivers. In the FR, the FR5 site stood out with the highest means for conductivity, TDS, salinity, TSS and apparent colour (Table 5). The values of true colour found at AR sites are typical for colourless or light waters, in opposition to those observed in the FR, especially at the downstream sites (FR4 to FR6) in summer (2013/14) (Table 5).

The pH was slightly acid in both rivers (especially in the AR) and, in the FR, this parameter was significantly lower in winter than in spring and summer (Table 5). Although AR sites presented a good ecological status considering the concentrations of nitrate and ammonium ion, some sites (AR1, AR2 and AR4) had a less than good ecological status due to their high phosphorus content, with the highest mean values occurring in winter, spring and summer of 2014 compared to spring and summer of 2013 (Table 5). The nitrites' content revealed levels of critical pollution (NO_2^- ≥ 1 mg/L) in the summer of 2013 (Table 5). At the FR sites, high levels of ammonium ion (FR4 to FR6), total phosphorus (all sites), nitrates (FR3, FR5 and FR6) and nitrites (all sites) were observed. In general, the highest levels of nutrients occurred in spring and summer (2013/14) compared to autumn and winter, and at the downstream sites (FR4 to FR6) compared to the upstream sites (FR1 and FR2; Table 5).

Table 5.

Spatial and seasonal variations of the water physico-chemical parameters determined in the Âncora and Ferreira rivers. Mean ± standard deviation of water temperature (Temp, °C), pH, dissolved oxygen concentration (DO, mg O_2/L), percent saturation of dissolved oxygen (% DO), chemical oxygen demand (COD, mg/L), conductivity (Cond, µS/cm), salinity (Sal, PSU), total dissolved solids (TDS, mg/L), total suspended solids (TSS, mg/L), apparent colour (App col, PCU), true colour (True col, PCU), total phosphorus (P, mg/L), nitrates (NO_3^-, mg/L), nitrites (NO_2^-, mg/L) and ammonium ion (NH_4^+, mg/L) are presented. Different letters identify significant differences ($p < 0.05$) between sampling sites or seasons, as indicated by post-hoc pairwise Wilcoxon rank sum tests.

	Âncora River									
	Sampling Site					Season				
	AR1	AR2	AR3	AR4	AR5	Summer /13	Autumn /13	Winter /14	Spring /14	Summer /14
Temp	15.06 ± 3.50	15.37 ± 2.66	15.10 ± 2.52	15.29 ± 2.80	15.40 ± 2.77	17.49 ± 0.69^{d}	14.38 ± 1.74^{a}	11.49 ± 3.13^{ab}	16.12 ± 1.45^{bc}	16.45 ± 0.90^{c}
pH	5.67 ± 0.62	5.42 ± 0.26	5.75 ± 0.41	5.71 ± 0.40	5.72 ± 0.34	5.73 ± 0.54	5.75 ± 0.65	5.51 ± 0.27	5.50 ± 0.27	5.67 ± 0.41
DO	8.30 ± 1.99	8.91 ± 1.87	8.20 ± 2.03	8.43 ± 2.55	8.88 ± 2.53	7.32 ± 2.34^{bc}	8.80 ± 1.16^{ac}	10.75 ± 0.84^{a}	6.96 ± 2.03^{b}	8.98 ± 1.50^{bc}
% DO	83.81 ± 17.97	89.15 ± 17.95	83.16 ± 19.66	85.88 ± 21.32	90.79 ± 20.65	76.36 ± 25.16^{a}	86.40 ± 9.56^{a}	99.40 ± 9.30^{b}	78.20 ± 21.39^{a}	92.38 ± 13.64^{ab}
COD	8.62 ± 12.93	12.69 ± 16.64	9.62 ± 13.88	9.62 ± 11.50	8.69 ± 11.65	10.73 ± 16.92	2.60 ± 4.35	20.70 ± 18.82	9.73 ± 8.42	6.67 ± 7.44
Cond	34.79 ± 39.38^{a}	40.50 ± 10.78^{b}	45.21 ± 9.32^{b}	45.71 ± 10.62^{b}	51.00 ± 10.33^{b}	52.07 ± 12.90^{a}	33.20 ± 9.31^{ab}	38.40 ± 13.88^{b}	52.40 ± 34.24^{ab}	37.87 ± 9.58^{b}
Sal	0.02 ± 0.02^{a}	0.02 ± 0.00^{b}	0.02 ± 0.00^{bc}	0.02 ± 0.01^{bc}	0.02 ± 0.01^{c}	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.02	0.02 ± 0.00
TDS	17.86 ± 19.69	20.14 ± 5.50	22.43 ± 4.47	22.93 ± 5.24	25.43 ± 5.06	25.87 ± 6.33^{a}	16.70 ± 4.64^{ab}	19.80 ± 6.63^{b}	26.27 ± 17.24^{ab}	18.87 ± 4.60^{b}
TSS	0.76 ± 0.90	1.76 ± 1.98	1.54 ± 3.94	1.50 ± 2.01	1.20 ± 2.04	1.16 ± 1.78	0.89 ± 0.72	2.95 ± 4.01	1.07 ± 1.67	0.53 ± 0.92
App col	9.64 ± 22.62	5.14 ± 13.41	5.71 ± 13.94	5.86 ± 12.15	4.43 ± 7.79	1.40 ± 3.20	19.10 ± 27.13	8.00 ± 16.12	1.27 ± 2.89	5.33 ± 10.36
True col	2.36 ± 7.46	0.07 ± 0.27	0.71 ± 1.73	0.21 ± 0.80	1.21 ± 3.38	0.00 ± 0.00	2.00 ± 3.80	0.13 ± 0.35	0.00 ± 0.00	2.80 ± 7.25
P	0.18 ± 0.13	0.23 ± 0.40	0.10 ± 0.07	0.20 ± 0.29	0.09 ± 0.10	0.08 ± 0.06^{ab}	0.07 ± 0.07^{b}	0.19 ± 0.28^{ac}	0.25 ± 0.40^{abc}	0.18 ± 0.09^{c}
NO_3^-	8.12 ± 3.41	8.24 ± 2.85	10.03 ± 4.55	8.74 ± 1.93	7.84 ± 2.15	9.81 ± 1.78^{a}	8.79 ± 5.38^{ab}	8.40 ± 2.52^{ab}	6.85 ± 2.27^{b}	9.19 ± 3.13^{ab}
NO_2^-	0.67 ± 2.49	0.53 ± 1.96	0.60 ± 2.23	0.13 ± 0.44	0.58 ± 2.14	2.33 ± 3.74^{a}	0.00 ± 0.01^{b}	0.01 ± 0.02^{ab}	0.01 ± 0.02^{ab}	0.00 ± 0.00^{b}
NH_4^+	0.46 ± 0.35	0.39 ± 0.17	0.26 ± 0.16	0.51 ± 0.58	0.29 ± 0.22	0.31 ± 0.20^{ab}	0.38 ± 0.08^{a}	0.39 ± 0.30^{ab}	0.25 ± 0.16^{b}	0.58 ± 0.59^{ab}

(Continuation of Table 5)

	Ferreira River										
	Sampling Site						Season				
	FR1	FR2	FR3	FR4	FR5	FR6	Summer /13	Autumn /13	Winter /14	Spring /14	Summer /14
Temp	16.71 ± 3.64	16.84 ± 3.63	17.30 ± 3.97	17.82 ± 4.40	17.59 ± 4.03	17.52 ± 4.22	20.43 ± 1.41^{a}	12.70 ± 2.39^{b}	13.70 ± 1.33^{b}	17.64 ± 2.25^{c}	20.49 ± 3.38^{a}
pH	6.12 ± 0.50	6.20 ± 0.50	6.03 ± 0.52	6.40 ± 0.60	6.40 ± 0.63	5.98 ± 0.50	6.38 ± 0.51^{a}	6.19 ± 0.27^{bc}	5.41 ± 0.25^{b}	6.32 ± 0.36^{ac}	6.64 ± 0.29^{a}
DO	8.23 ± 1.95	8.67 ± 2.92	8.38 ± 2.22	8.23 ± 2.48	8.34 ± 2.57	7.95 ± 2.64	5.82 ± 1.71^{a}	8.53 ± 0.55^{bc}	11.77 ± 1.37^{c}	8.60 ± 0.66^{b}	6.86 ± 1.20^{a}
% DO	86.14 ± 18.18	91.06 ± 27.39	87.63 ± 20.36	84.28 ± 22.17	85.69 ± 22.24	82.34 ± 23.49	64.72 ± 17.8^{a}	82.34 ± 8.84^{bc}	111.90 ± 14.15^{c}	89.66 ± 5.39^{b}	78.15 ± 17.70^{ab}
COD	2.62 ± 5.66	13.00 ± 13.46	6.08 ± 10.91	11.85 ± 15.01	13.23 ± 27.72	9.77 ± 13.55	18.11 ± 24.25	5.50 ± 12.55	11.08 ± 10.42	3.17 ± 9.34	8.50 ± 12.38
Cond	113.07 ± 14.67^{a}	123.29 ± 16.28^{ab}	149.43 ± 43.05^{bc}	207.00 ± 103.42^{c}	233.79 ± 109.90^{c}	211.29 ± 97.63^{c}	274.78 ± 126.31^{a}	140.25 ± 26.87^{bc}	117.83 ± 30.85^{c}	151.94 ± 31.86^{ab}	169.17 ± 57.62^{ab}
Sal	0.05 ± 0.01^{a}	0.06 ± 0.01^{a}	0.07 ± 0.02^{ab}	0.10 ± 0.05^{b}	0.11 ± 0.05^{b}	0.10 ± 0.05^{b}	0.13 ± 0.06^{a}	0.07 ± 0.01^{b}	0.06 ± 0.02^{b}	0.07 ± 0.01^{b}	0.08 ± 0.03^{ab}
TDS	56.43 ± 7.33^{a}	61.71 ± 8.06^{a}	74.70 ± 21.69^{ab}	103.43 ± 51.59^{b}	116.79 ± 54.84^{b}	105.64 ± 48.78^{b}	137.28 ± 63.00^{a}	70.00 ± 13.33^{bcd}	58.83 ± 15.50^{d}	75.89 ± 16.08^{c}	84.78 ± 28.73^{abc}
TSS	1.28 ± 1.34^{a}	3.38 ± 3.79^{ab}	2.59 ± 2.67^{ab}	3.02 ± 3.17^{a}	16.65 ± 23.45^{b}	5.21 ± 4.57^{ab}	4.18 ± 3.56	9.16 ± 26.11	5.39 ± 4.57	3.11 ± 3.31	6.21 ± 9.56
App col	13.86 ± 11.15^{a}	23.57 ± 17.06^{a}	17.07 ± 12.36^{a}	35.14 ± 27.44^{ab}	61.07 ± 33.87^{b}	35.57 ± 26.43^{ab}	38.83 ± 28.22	21.92 ± 16.41	27.50 ± 28.22	30.11 ± 32.26	33.83 ± 26.30
True col	3.64 ± 4.70	5.43 ± 7.49	5.93 ± 7.71	10.50 ± 16.07	11.71 ± 12.87	9.79 ± 12.08	12.61 ± 15.83^{ab}	2.67 ± 3.28^{a}	4.22 ± 6.23^{ab}	5.17 ± 7.78^{ab}	12.78 ± 11.73^{b}
P	0.14 ± 0.12	0.23 ± 0.18	0.28 ± 0.21	0.59 ± 0.48	0.56 ± 0.44	0.51 ± 0.42	0.59 ± 0.46^{a}	0.21 ± 0.10^{b}	0.11 ± 0.15^{b}	0.37 ± 0.23^{a}	0.58 ± 0.44^{a}
NO_3^-	20.97 ± 7.03^{ab}	18.98 ± 3.64^{a}	25.50 ± 8.62^{ab}	23.93 ± 6.72^{ab}	27.86 ± 8.71^{b}	25.88 ± 7.95^{ab}	29.25 ± 8.58^{a}	22.61 ± 4.33^{ab}	18.93 ± 5.11^{c}	20.52 ± 4.32^{bc}	27.54 ± 8.75^{abc}
NO_2^-	0.58 ± 2.04^{a}	0.97 ± 3.46^{a}	3.27 ± 7.69^{abc}	8.52 ± 15.86^{bc}	9.80 ± 16.74^{abc}	6.97 ± 11.16^{bc}	7.29 ± 8.18^{a}	0.20 ± 0.19^{c}	0.13 ± 0.20^{bc}	7.27 ± 11.69^{ab}	8.59 ± 18.82^{abc}
NH_4^+	0.24 ± 0.13^{a}	0.71 ± 0.61^{ab}	0.64 ± 0.51^{abd}	3.40 ± 2.77^{c}	2.37 ± 2.16^{bcd}	1.45 ± 1.03^{bcd}	2.64 ± 2.95	1.09 ± 0.61	0.53 ± 0.42	1.00 ± 0.65	1.94 ± 2.06

Note: limit values established for the “good” ecological status in Northern Portuguese rivers: DO ≥ 5 mg O_2/L (80% samples), % DO: 60 – 120 % (80% samples), P ≤ 0.10 mg/L (mean); NO_3^- ≤ 25 mg/L (mean); NH_4^+ ≤1 mg/L (80% samples).

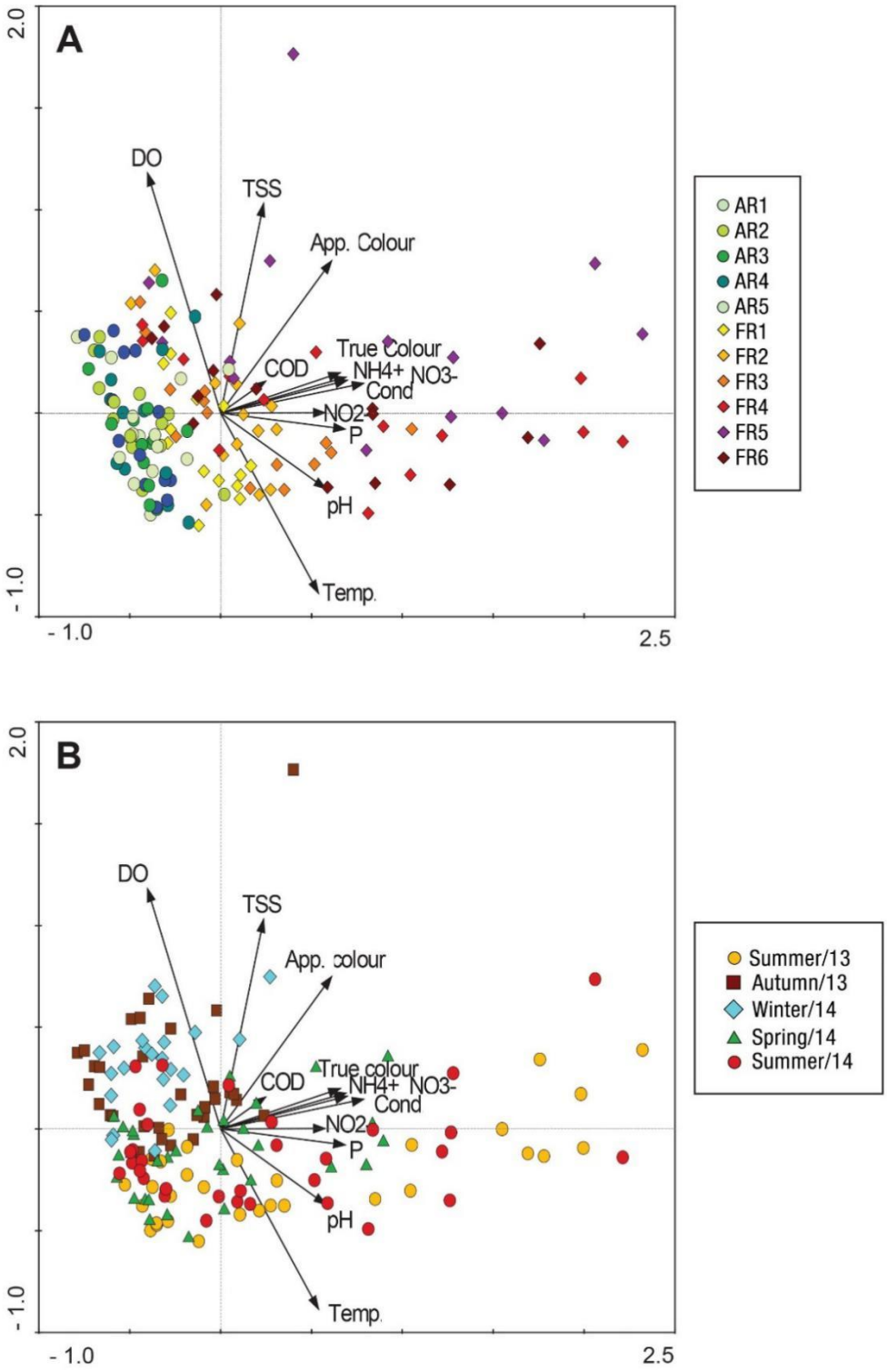

Fig. 4. PCA of the monthly determined (from July 2013 to September 2014) water physico-chemical parameters (Temp, water temperature; pH; DO, dissolved oxygen concentration; COD, chemical oxygen demand; pH, Cond, conductivity; App colour, apparent colour; True colour; TSS, total suspended solids; NH_4^+, ammonium ion; NO_3^-, nitrates; NO_2^-, nitrites; P, total phosphorus) determined in the Âncora (AR) and Ferreira (FR) rivers, with (A) samples marked according to sampling sites (AR1 to AR5 and FR1 to FR6) and (B) seasons.

The horizontal axis of the PCA of the water physico-chemical parameters clearly discriminated between rivers, mainly separating the downstream sites of the FR from the AR sites (Fig. 4A). This axis explained 43.8% of the total variability in dataset. Nutrients (nitrates, nitrites, phosphorus and to a lesser extent ammonium ion), conductivity, true colour and pH were the parameters that contributed most to this discrimination (Fig. 4A). The vertical axis was driven by seasonality. It separated autumn and winter samples from spring and summer (2013/14) samples, explaining 10.8% of the variability. DO, TSS and water temperature were the parameters that contributed most to the discrimination between seasons (Fig. 4B).

3.4.2. Hydromorphological quality elements and sediment analysis

The hydrologic parameters (discharge and flow velocity), some of the sediment granulometric classes percentages, the habitat quality indices determined and species richness in the riparian zone showed significant differences between AR and FR (% very coarse sand, $p = 0.0112$; % medium + fine + very fine sand, $p = 0.0013$; % silt and clay, $p = 0.0011$; HQA, $p = 0.0001$; HMS, $p < 0.0001$; QBR, $p = 0.0003$; GQC, $p = 0.0003$; RVI, $p = 0.0015$; species richness, $p = 0.0194$). Overall, FR sites presented higher discharge, flow velocity, percentages of finer sediments (medium sand to clay), HMS index scores and species richness in the riparian zone compared to the AR sites. In opposition, AR sites had higher percentages of very coarse sand and higher scores of HQA, QBR, GQC and RVI indices than FR sites (Table 6).

Table 6.

Spatial and seasonal variations of the hydrological (discharge and flow velocity) and sediment (organic matter content in sediment and granulometry) parameters determined in the Âncora and Ferreira rivers. Mean ± standard deviation of discharge (m^3/s), flow velocity (m/s), organic matter content in sediment (% OM) and sediment size-classes according to the Wentworth scale (expressed as percentage of the total weight) are presented. Different letters identify significant differences ($p < 0.05$) between sampling sites or seasons, as indicated by post-hoc pairwise Wilcoxon rank sum tests.

Âncora River	Sampling Site					Season/Year			
	AR1	AR2	AR3	AR4	AR5	Summer /13	Autumn /13	Spring /14	Summer /14
Discharge	12.53 ± 14.06	34.26 ± 58.83	17.19 ± 26.79	18.20 ± 27.88	48.56 ± 77.58	10.27 ± 5.78[a]	87.26 ± 54.33[b]	5.98 ± 3.49[a]	1.07 ± 0.68[c]
Flow velocity	13.94 ± 11.69	14.29 ± 15.34	11.31 ± 14.88	13.85 ± 11.97	34.06 ± 36.98	8.82 ± 5.65[a]	43.73 ± 25.27[b]	13.52 ± 6.62[a]	3.89 ± 2.77[a]
% OM	1.34 ± 0.90	2.45 ± 1.62	1.79 ± 0.25	1.71 ± 0.09	1.22 ± 0.43	1.57 ± 0.70	2.31 ± 1.36	1.73 ± 0.60	1.20 ± 0.63
% Pebble and Granule	18.77 ± 9.72	38.42 ± 23.64	39.24 ± 25.79	35.26 ± 24.28	39.96 ± 26.65	38.21 ± 8.66[a]	6.46 ± 3.76[b]	46.00 ± 21.59[a]	46.64 ± 19.36[a]
% Very coarse sand	39.69 ± 8.07	31.74 ± 1.76	34.23 ± 9.69	38.39 ± 11.65	42.69 ± 16.36	40.22 ± 7.09	46.04 ± 12.78	33.23 ± 7.30	29.90 ± 6.19
% Coarse sand	30.25 ± 6.14	23.59 ± 20.44	18.82 ± 14.48	22.05 ± 12.07	15.52 ± 10.23	16.25 ± 4.37	36.85 ± 11.49	16.92 ± 12.68	18.17 ± 11.39
% Medium, fine and very fine sand	11.11 ± 2.96	6.09 ± 5.09	7.57 ± 7.64	4.20 ± 3.93	1.73 ± 1.16	5.20 ± 5.76	10.57 ± 5.84	3.64 ± 4.31	5.15 ± 3.44
% Silt and clay	0.19 ± 0.05	0.15 ± 0.08	0.15 ± 0.09	0.10 ± 0.05	0.09 ± 0.12	0.12 ± 0.08	0.08 ± 0.07	0.21 ± 0.04	0.14 ± 0.09
Ferreira River	Sampling Site						Season/Year			
	FR1	FR2	FR3	FR4	FR5	FR6	Summer /13	Autumn /13	Spring /14	Summer /14
Discharge	29.06 ± 45.68	65.99 ± 105.18	40.21 ± 58.96	95.19 ± 130.72	57.13 ± 81.74	57.14 ± 74.60	22.56 ± 9.83[a]	181.27 ± 69.29[b]	13.96 ± 8.35[a]	12.03 ± 9.32[a]
Flow velocity	17.88 ± 18.78	54.31 ± 45.73	39.43 ± 38.67	67.36 ± 57.53	46.24 ± 43.26	44.69 ± 29.66	22.70 ± 11.59[a]	102.58 ± 36.41[b]	29.27 ± 12.00[a]	25.39 ± 12.12[a]
% OM	0.94 ± 0.09	1.41 ± 0.70	1.25 ± 0.33	1.14 ± 0.18	2.08 ± 0.94	1.55 ± 0.62	1.81 ± 0.85	1.41 ± 0.28	1.21 ± 0.54	1.14 ± 0.59
% Pebble and granule	7.55 ± 6.75	28.12 ± 13.44	41.36 ± 17.44	34.79 ± 13.37	25.60 ± 12.00	36.89 ± 31.02	22.57 ± 10.41	12.87 ± 8.42	37.42 ± 18.66	43.35 ± 21.08
% Very coarse sand	37.95 ± 10.57	27.58 ± 5.85	23.83 ± 7.81	28.42 ± 4.71	31.15 ± 11.79	30.21 ± 6.68	26.31 ± 5.69	29.77 ± 4.79	32.89 ± 13.40	30.44 ± 8.66
% Coarse sand	32.90 ± 12.00	22.76 ± 7.71	13.94 ± 3.70	19.53 ± 4.91	24.83 ± 5.21	16.15 ± 8.74	20.34 ± 3.51	25.49 ± 6.85	20.07 ± 9.43	20.85 ± 14.86
% medium, fine and very fine sand	14.77 ± 7.16	15.11 ± 7.36	14.81 ± 2.65	10.93 ± 6.90	12.13 ± 9.96	10.32 ± 9.47	17.80 ± 0.94[a]	19.64 ± 3.06[a]	9.40 ± 6.23[b]	5.21 ± 3.17[b]
% Silt and clay	6.82 ± 7.59	6.43 ± 7.01	6.06 ± 6.91	6.33 ± 7.19	6.30 ± 7.07	6.43 ± 7.32	12.99 ± 0.49[a]	12.22 ± 0.49[a]	0.22 ± 0.19[b]	0.15 ± 0.09[b]

In both rivers, significant seasonal differences were found for the hydrologic parameters (discharge and flow velocity), with higher values occurring in autumn than in spring and summer (2013/14) and also for some sediment size-classes (Table 6). In the

AR, the percentage of pebble and granule was significantly lower in autumn than in spring and summer (2013/14) and in the FR, the percentages of medium, fine and very fine sand and of silt and clay were significantly lower in summer and autumn of 2013 than in spring and summer of 2014 (Table 6). Significant spatial differences were found for all habitat quality indices determined (AR: HQA p = 0.0182, HMS p = 0.0072, QBR p = 0.0007, GQC p = 0.0055, RVI p = 0.0229; FR: HQA p =0.0149, HMS p = 0.0045, QBR p = 0.0003, GQC p = 0.0005, RVI p = 0.0229), in both rivers, and for the species richness in the riparian zone, in the AR.

Table 7.

Scores of the habitat quality indices (Habitat Quality Assessment, HQA; Habitat Modification Score, HMS; Riparian Forest Quality, QBR; Channel Quality Degree, GQC; and Riparian Vegetation, RVI) determined at the sampling sites of the Âncora (AR1 to AR5) and Ferreira (FR1 to FR6) rivers in different seasons. Corresponding quality classes of all indices' scores (only class I was considered for HQA and HMS indices, since in Portugal, limit values both indices were only defined for the class I; class I to V for QBR, GQC and RVI indices), the categories of artificialization of river channel morphology according to the HMS index (categories 1 to 4) and the species richness in the riparian zone, are also given. n.d.: not determined.

Season /Year	Site	HQA Score (Class)	HMS Score (Category; Class)	QBR Score (Class)	GQC Score (Class)	RVI	
						Score (Class)	Species richnessspecies
Summer /13	AR1	n.d.	n.d.	15 (V)	31 (I)	n.d.	n.d.
	AR2	n.d.	n.d.	25 (V)	27 (II)	n.d.	n.d.
	AR3	n.d.	n.d.	100 (I)	32 (I)	n.d.	n.d.
	AR4	n.d.	n.d.	65 (III)	31 (I)	n.d.	n.d.
	AR5	n.d.	n.d.	45 (IV)	28 (II)	n.d.	n.d.
Autumn /13	AR1	64 (I)	420 (3)	15 (V)	32 (I)	1.25 (I)	31
	AR2	72 (I)	885 (4)	25 (V)	27 (II)	1.33 (I)	53
	AR3	65 (I)	0 (1; I)	100 (I)	29 (II)	1.00 (I)	29
	AR4	61 (I)	715 (4)	65 (III)	32 (I)	1.17 (I)	36
	AR5	65 (I)	845 (4)	45 (IV)	29 (II)	1.00 (I)	36
Spring /14	AR1	68 (I)	420 (3)	15 (V)	30 (II)	1.25 (I)	35
	AR2	71 (I)	885 (4)	25 (V)	28 (II)	1.08 (I)	62
	AR3	66 (I)	0 (1; I)	100 (I)	29 (II)	0.75 (I)	44
	AR4	60 (I)	715 (4)	65 (III)	29 (II)	1.08 (I)	34
	AR5	62 (I)	845 (4)	45 (IV)	27 (II)	1.08 (I)	50
Summer /14	AR1	68 (I)	420 (3)	15 (V)	32 (I)	1.25 (I)	29
	AR2	70 (I)	885 (4)	25 (V)	27 (II)	1.17 (I)	51
	AR3	66 (I)	0 (1; I)	100 (I)	29 (II)	0.83 (I)	31
	AR4	60 (I)	715 (4)	65 (III)	30 (II)	1.08 (I)	36
	AR5	65 (I)	845 (4)	45 (IV)	28 (II)	0.92 (I)	40
Summer /13	FR1	n.d.	n.d.	15 (V)	19 (IV)	n.d.	n.d.
	FR2	n.d.	n.d.	15 (V)	29 (II)	n.d.	n.d.
	FR3	n.d.	n.d.	25 (V)	24 (III)	n.d.	n.d.
	FR4	n.d.	n.d.	25 (V)	30 (II)	n.d.	n.d.
	FR5	n.d.	n.d.	15 (V)	27 (II)	n.d.	n.d.
	FR6	n.d.	n.d.	25 (V)	23 (III)	n.d.	n.d.
Autumn /13	FR1	51 (I)	1045 (4)	15 (V)	19 (IV)	0.5 (III)	40
	FR2	43	1180 (4)	15 (V)	26 (II)	0.67 (II)	50
	FR3	50 (I)	1000 (4)	25 (V)	24 (III)	0.42 (III)	50
	FR4	70 (I)	830 (4)	25 (V)	30 (II)	1.00 (I)	52
	FR5	41	1285 (4)	15 (V)	26 (II)	0.83 (I)	55
	FR6	39	1010 (4)	25 (V)	23 (III)	0.08 (V)	34
Spring /14	FR1	50 (I)	1045 (4)	15 (V)	19 (IV)	0.33 (IV)	61
	FR2	41	1180 (4)	15 (V)	30 (II)	0.67 (II)	52
	FR3	49 (I)	1000 (4)	25 (V)	24 (III)	0.33 (IV)	65
	FR4	65 (I)	830 (4)	25 (V)	30 (II)	0.83 (I)	62
	FR5	38	1285 (4)	15 (V)	26 (II)	0.42 (III)	45
	FR6	39	1010 (4)	25 (V)	21 (III)	0.25 (IV)	40
Summer /14	FR1	56 (I)	1045 (4)	15 (V)	19 (IV)	0.42 (III)	47
	FR2	40	1180 (4)	15 (V)	30 (II)	0.67 (II)	56
	FR3	45	1000 (4)	25 (V)	23 (III)	0.50 (III)	50
	FR4	70 (I)	830 (4)	25 (V)	30 (II)	0.83 (I)	47
	FR5	48	1285 (4)	15 (V)	26 (II)	0.25 (IV)	46
	FR6	45	1010 (4)	25 (V)	23 (III)	0.17 (V)	28

Note: *HQA and HMS ecological quality classes for "small sized streams of North of Portugal"*: HQA score ≥ 46, class I (high ecological quality, i.e., high habitat diversity); HMS score ≤ 16, class I (high ecological quality, i.e., no artificial modifications in the river channel morphology). *HMS categories of artificialization of river channel morphology*: score ≤ 16, category 1 (pristine/semi-natural); score 17-199, category 2 (predominantly non-modified); score 200-499, category 3 (obviously modified); score 500-1399, category 4 (significantly modified); score ≥ 1400, category 5 (severely modified). *QBR classes of riparian habitat quality*: score ≥ 95, class I (riparian habitat in natural condition); score 75-90, class II (some disturbance, good quality); score 55-70, class III (important disturbance, fair quality); score 30-50, class IV (strong alteration, poor quality); score ≤ 25, class V (extreme alteration, bad quality). *GQC channel quality classes*: score ≥ 31, class I (unchanged channel, natural state); score 26-30, class II (slightly disturbed channel); score 20-25, class III (onset of significant change in the channel); score 14-19, class IV (major channel change); score 8-13, class V (completely changed channel). *RVI ecological quality classes for North region of Portugal*: score ≥ 0.67, class I (high ecological

quality); score 0.50-0.66, class II (good ecological quality); score 0.33-0.49, class III (moderate ecological status); score 0.16-0.32, class IV (poor ecological quality); score ≤ 15, class V (bad ecological quality).

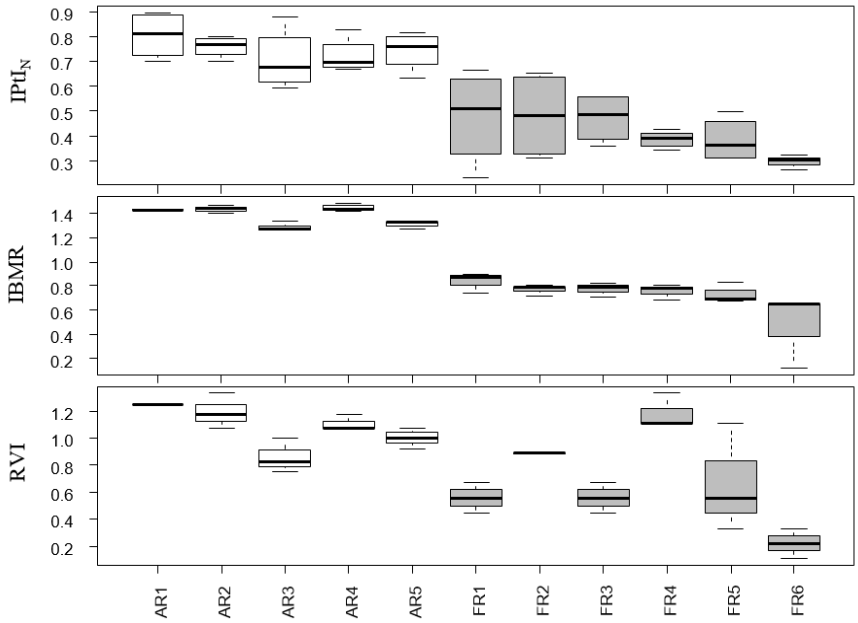

Fig. 5. Box-plots of the North Invertebrate Portuguese Index ($IPtI_N$) and Macrophyte Biological Index for Rivers (IBMR) recommended by the Water Framework Directive and the macrophyte-based index of biotic integrity Riparian Vegetation Index (RVI) seasonally determined ($IPtI_N$ in summer and autumn 2013 and spring and summer 2014; IBMR and RVI in autumn 2013 and spring and summer of 2014) at the sampling sites of the Âncora (AR1 to AR5) and Ferreira (FR1 to FR6) rivers. The length of each box shows the range within which the central 50% of the values fall, with the box edges at the first and third quartiles.

The RHS indices (HQA and HMS) indicated that all AR sites had high physical habitat heterogeneity (HQA, class I) but only AR3 presented bed and river banks in natural condition (HMS category 1; class I) (Table 7). In the remaining sites the physical structure of the channel was obviously (category 3; AR1) or significantly modified (category 4; AR2, AR4 and AR5) (Table 7). Channel quality of all AR sites, evaluated by the GQC index, was unmodified (class I) or slightly disturbed (class II) (Table 7). According to the QBR index, the riparian habitat was in natural condition only in the AR3 site (class I), while other sites showed riparian zones with fair (class III; AR4), poor (class IV; AR5), or very bad quality (class V; AR1 e AR2), mainly due to the degree of coverage of the riparian zone and the structure and quality of the plant cover (Table 7). Although the RVI multimetric index final score indicated high quality of the riparian vegetation (class I) at all AR sites (Table 7, Fig. 5), almost all sites had bad quality (except AR4 in summer (13/14) and autumn and AR2 in autumn, with moderate quality) regarding the metrics proportion and coverage of alien species (e.g.

species found in more than one sampling site: *Acacia* sp., *Bidens frondosa*, *Conyza bilbaoana*, *Conyza sumatrensis*, *Crocosmia* x *crocosmiflora*, *Digilaria sanguinalis*, *Eucalyptus globulus*, *Phytolacca americana* and invasive *Vitis* taxa and nothotaxa). AR5 was the site with the highest proportion of alien species in all seasons (AR5: 14 to 25%; the remaining sites: 5.5 to 13.7%) and AR3 the site with the densest coverage of alien species (AR3: 16 to 17%: remaining sites: 1.5 to 14.5%).

In the FR, HQA and HMS results indicated that only FR1, FR3 and FR4 sites had high physical habitat heterogeneity (HQA, class I) and that the channel´s physical structure of all sampling sites was significantly modified (HMS, category 4) (Table 7). According to the QBR and GQC indices, the riparian vegetation of FR sites was extremely altered (QBR, class V) and the channel was slightly disturbed (GQC, class II; FR2, FR4 and FR5), starting to show significantly alterations (GQC, class III; FR3 and FR6) or strongly altered (GQC, class IV; FR1) (Table 7). The riparian vegetation quality, evaluated by the RVI index, was high or good at FR4 and FR2 (class I and II, respectively), moderate or poor at FR1, FR3 and FR5 (classes III and IV; except for FR5's high quality in autumn) and poor or bad at FR6 (classes IV and V, respectively) (Table 7, Fig. 5). In general, RVI results indicated FR1, FR3, FR5 and FR6 as showing lower proportion of endemic species (in all sites in spring and summer and in FR6 in autumn), less weighted woody cover (all sites in all seasons) and *Carex elata* ssp. *reuterana cover* (FR6 in autumn, FR3 and FR5 in spring and all sites in summer). With regard to the metrics proportion and coverage of alien species (e.g. *Acacia* spp., *Amaranthus* spp., *Bidens frondosa*, *Calystegia sylvatica*, *Conyza* spp., *Cyperus eragrostis*, *Oenothera glazioviana*, *Paspalum paspalodes*, *Phytolacca americana*, *Solanum chenopodioides,* hybrid grapes), all FR sites showed bad quality in all seasons.

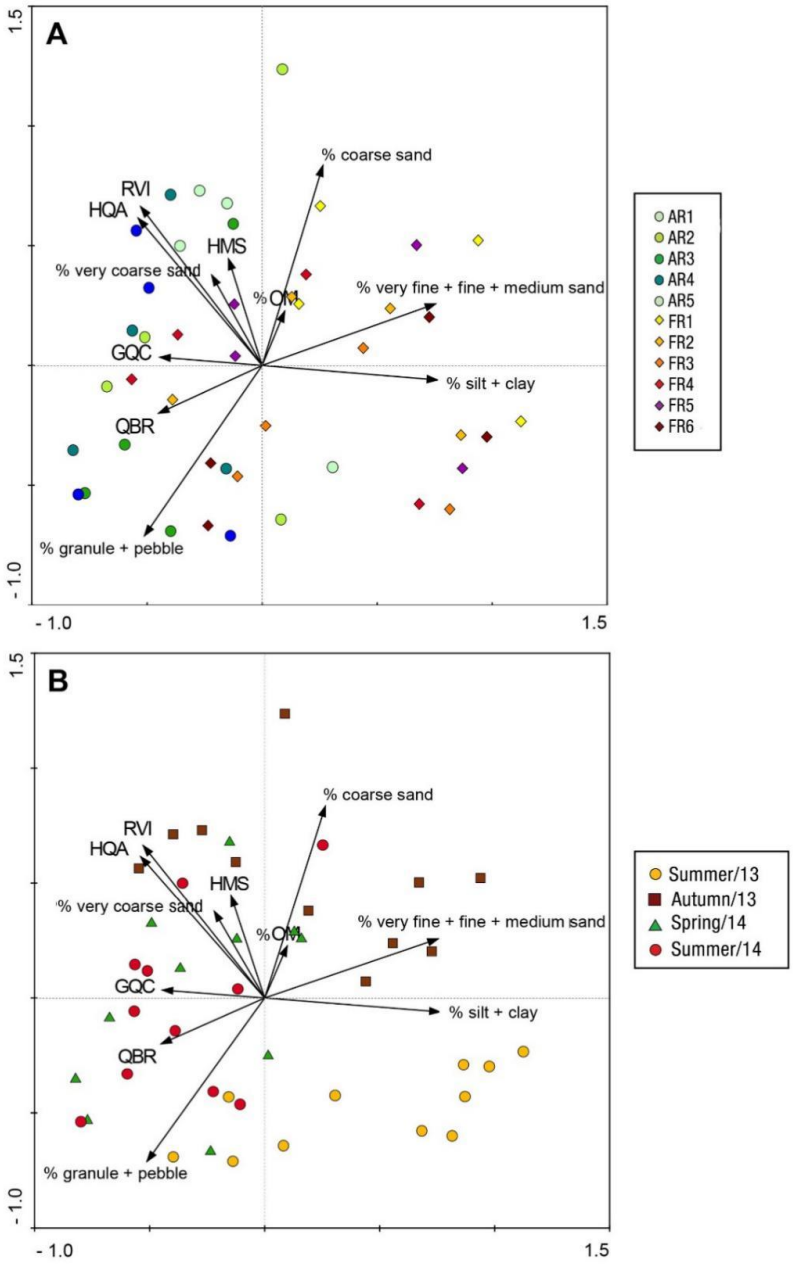

Fig. 6. PCA of the hydromorphological indices (HQA, Habitat Quality Assessment index; HMS, Habitat Modification Score index; QBR, Riparian Forest Quality index, GQC, Channel Quality Degree index; RVI, Riparian Vegetation index) and sediment parameters (organic matter content expressed as percentage of organic matter, % OM; sediment size-classes expressed as percentage of the total weight: % granule + pebble, % very coarse sand, % coarse sand, % very fine + fine + medium sand, % silt + clay) seasonally determined (HQA, HMS and RVI not determined in summer/13) in the Âncora (AR) and Ferreira (FR) rivers, with samples marked according to (A) sampling sites (AR1 to AR5; FR1 to FR6) and (B) seasons.

The horizontal axis of the PCA of the hydromorphological (HQA, HMS, QBR, GQC and RVI indices) and sediment parameters (organic matter content and sediment size-classes) discriminated between the two rivers, explaining 28.3% of the total variability (Fig. 6A). The percentages of finer sediments (medium sand to clay)

contributed most to this discrimination, and to a lesser extent the QBR, GQC RVI and HQA indices. The vertical axis discriminated between seasons, mainly summer (2013/14) from autumn, explaining 23.4% of the total variability. The percentages of granule and pebble and coarse sand were the parameters that contributed most to this discrimination (Fig. 6B).

3.4.3. Benthic macroinvertebrates

Significant differences between rivers were observed for Hydropsychidae individuals (%), number of families (No families) and Shannon-Wiener (H'), biotic (BBI and IBMWP) and multimetric ($IPtI_N$) indices (% Hydropsychidae, $p = 0.0021$; No. families, $p < 0.0001$; H', BBI, IBMWP and $IPtI_N$, $p < 0.0001$) (Table 8, Fig. 5). The % Hydropsychidae was higher in the FR, whereas the other metrics were higher in the AR (Table 8). The total number of organisms ($Log_{10}N$) and the equitability index (E) showed similar values in both rivers indicating that organisms were not equally distributed among different families (Table 8), with a clear dominance of certain taxa tolerant to organic pollution (e.g. Chironomidae family during spring and summer and the Oligochaeta class in autumn) over the most sensitive taxa (e.g. Ephemeroptera, Plecoptera, Trichoptera).

Significant differences were found for % Chironomidae in both rivers (AR $p = 0.0373$; FR $p = 0.0296$) and for the total number of organisms ($Log_{10}N$) only in the AR ($p = 0.0445$), between seasons, but not between sampling sites.

Table 8.

Total number of macroinvertebrate individuals ($Log_{10}N$, being N the total No. of individuals) and measures of richness (No families), composition (percentage of Ephemeroptera, Plecoptera and Trichoptera orders, % EPT; % Hydropsychidae, % Hydrop.; % Chironomidae, % Chir.), diversity (Pielou's equitability index, E; Shannon-Wiener index, H') and biotic (Biotic Belgian Index, BBI; Iberian Biological Monitoring Working Party Index, IBMWP) and multimetric (North Invertebrate Portuguese Index, $IPtI_N$) indices based on the benthic macroinvertebrate community determined at the sampling sites of the Âncora (AR1 to AR5) and Ferreira (FR1 to FR6) rivers in different seasons. Corresponding quality classes of the biotic and multimetric indices are also given.

Season /Year	Site	Log10 N	No Families	H'	E	% EPT	% Hidrop.	% Chir.	BBI Score (Class)	IBMWP Score (Class)	$IPtI_N$ Score (Class)
Summer /13	AR1	3.22	23	1.48	0.47	30.05	0.18	58.31	9 (I)	144 (I)	0.69 (II)
	AR2	3.20	26	1.30	0.40	9.40	0.00	50.66	10 (I)	151 (I)	0.69 (II)
	AR3	2.27	21	2.30	0.75	18.09	0.00	30.85	9 (I)	120 (I)	0.63 (III)
	AR4	3.00	24	0.96	0.30	8.21	0.00	80.61	10 (I)	136 (I)	0.66 (III)
	AR5	3.28	27	1.29	0.39	10.98	1.11	26.15	10 (I)	157 (I)	0.80 (II)
Autumn /13	AR1	2.37	25	2.52	0.78	20.00	0.00	14.47	10 (I)	153 (I)	0.73 (II)
	AR2	2.55	24	2.42	0.76	13.96	1.99	15.38	10 (I)	134 (I)	0.74 (II)
	AR3	2.14	16	1.76	0.63	8.70	1.45	28.26	9 (I)	100 (II)	0.58 (III)
	AR4	2.54	23	1.68	0.54	11.27	0.00	19.65	10 (I)	148 (I)	0.69 (II)
	AR5	2.16	15	1.83	0.68	12.41	0.00	11.72	8 (II)	95 (II)	0.62 (III)
Spring /14	AR1	3.01	37	2.30	0.64	56.63	0.29	16.96	10 (I)	207 (I)	0.86 (II)
	AR2	2.68	28	1.59	0.48	28.69	0.00	50.10	10 (I)	171 (I)	0.79 (II)
	AR3	2.88	28	1.90	0.57	31.50	0.00	43.01	9 (I)	193 (I)	0.86 (II)
	AR4	2.53	22	1.34	0.43	13.27	0.00	68.44	9 (I)	138 (I)	0.67 (III)
	AR5	2.83	26	1.92	0.59	28.68	0.30	14.12	10 (I)	162 (I)	0.73 (II)
Summer /14	AR1	3.46	34	1.37	0.39	10.75	0.07	69.29	10 (I)	206 (I)	0.88 (I)
	AR2	3.72	28	1.27	0.38	13.12	8.29	70.29	10 (I)	159 (I)	0.77 (II)
	AR3	2.47	17	2.10	0.74	20.20	0.00	26.26	10 (I)	107 (I)	0.69 (II)
	AR4	3.04	29	1.85	0.55	10.69	1.66	47.28	10 (I)	187 (I)	0.81 (II)
	AR5	2.74	24	1.80	0.57	11.64	0.18	53.64	10 (I)	151 (I)	0.77 (II)
Summer /13	FR1	3.14	21	1.00	0.33	7.50	0.07	71.88	9 (I)	115 (I)	0.56 (III)
	FR2	2.74	11	1.19	0.49	30.36	1.09	66.73	8 (I)	57 (III)	0.53 (III)
	FR3	3.16	16	1.28	0.46	35.99	10.05	60.01	9 (I)	84 (II)	0.47 (III)
	FR4	3.59	10	1.06	0.46	30.50	20.20	64.14	5 (III)	43 (III)	0.34 (IV)
	FR5	3.01	10	0.97	0.42	36.59	23.04	62.92	7 (II)	55 (III)	0.42 (IV)
	FR6	2.92	9	0.22	0.10	3.21	1.07	96.31	5 (III)	39 (III)	0.28 (IV)
Autumn /13	FR1	2.74	8	1.09	0.52	9.31	0.00	30.84	7 (II)	37 (III)	0.37 (IV)
	FR2	3.16	7	0.86	0.44	6.01	0.77	66.60	5 (III)	29 (IV)	0.29 (IV)
	FR3	3.72	8	0.92	0.44	4.44	0.34	13.44	5 (III)	35 (IV)	0.31 (IV)
	FR4	3.97	9	0.49	0.22	4.33	0.76	5.62	5 (III)	39 (III)	0.29 (IV)
	FR5	2.83	10	1.46	0.64	33.63	11.70	23.11	5 (III)	43 (III)	0.36 (IV)
	FR6	3.24	6	0.41	0.23	3.96	0.00	6.02	5 (III)	24 (IV)	0.23 (IV)
Spring /14	FR1	2.75	15	0.67	0.25	10.20	0.00	86.23	8 (II)	88 (II)	0.50 (III)
	FR2	3.52	12	0.65	0.26	3.16	0.00	77.85	6 (III)	45 (III)	0.27 (IV)
	FR3	2.94	13	1.27	0.50	39.03	14.20	57.16	8 (II)	65 (II)	0.47 (III)
	FR4	2.66	12	1.41	0.57	36.66	31.89	43.38	6 (III)	52 (III)	0.37 (IV)
	FR5	2.72	8	1.60	0.77	31.23	16.67	43.68	5 (III)	24 (IV)	0.26 (IV)
	FR6	2.77	7	1.14	0.59	13.30	10.10	65.49	5 (III)	24 (IV)	0.26 (IV)
Summer /14	FR1	2.48	7	0.55	0.28	11.33	0.33	86.33	5 (III)	22(IV)	0.20 (V)
	FR2	3.72	16	1.10	0.40	2.45	0.36	30.71	8 (II)	79 (II)	0.56 (III)
	FR3	2.43	10	1.14	0.50	16.04	2.61	64.55	5 (III)	46 (III)	0.36 (IV)
	FR4	2.57	10	1.31	0.57	17.30	7.57	61.89	5 (III)	41 (III)	0.32 (IV)
	FR5	3.10	7	1.30	0.67	34.48	2.56	49.60	5 (III)	24 (IV)	0.27 (IV)
	FR6	3.11	8	1.19	0.57	15.27	1.01	34.19	5 (III)	27 (IV)	0.26 (IV)

Note: *BBI quality classes*: score 9-10, class I (lightly or unpolluted water); score 7-8, class II (slightly polluted water); score 5-6, class III (moderately polluted water, critical situation); score 3-4, class IV (heavily polluted water); score 0-2, class V (very heavily polluted water). *IBMWP quality classes* : score > 100, class I (good quality; score 101-150, pristine waters;

score > 150, non-polluted, or not noticeably altered system); score 61-100, class II (acceptable quality, i.e., evidence of effects of mild pollution); score 36-60, class III (dubious quality, i.e., polluted waters or altered system); score 16-35, class IV (critical quality, i.e., very polluted waters or very altered system); score ≤ 15, class V (very critical quality, i.e., strongly polluted waters or strongly altered system). *IPtI$_N$ ecological quality classes for "small sized streams of North of Portugal"*: score ≥ 0.87, class I (high ecological status); score 0.68-0.86, class II (good ecological status); score 0.44-0.67, class III (moderate ecological status); score 0.22-0.43, class IV (poor ecological status); score ≤ 0.21, class V (bad ecological status).

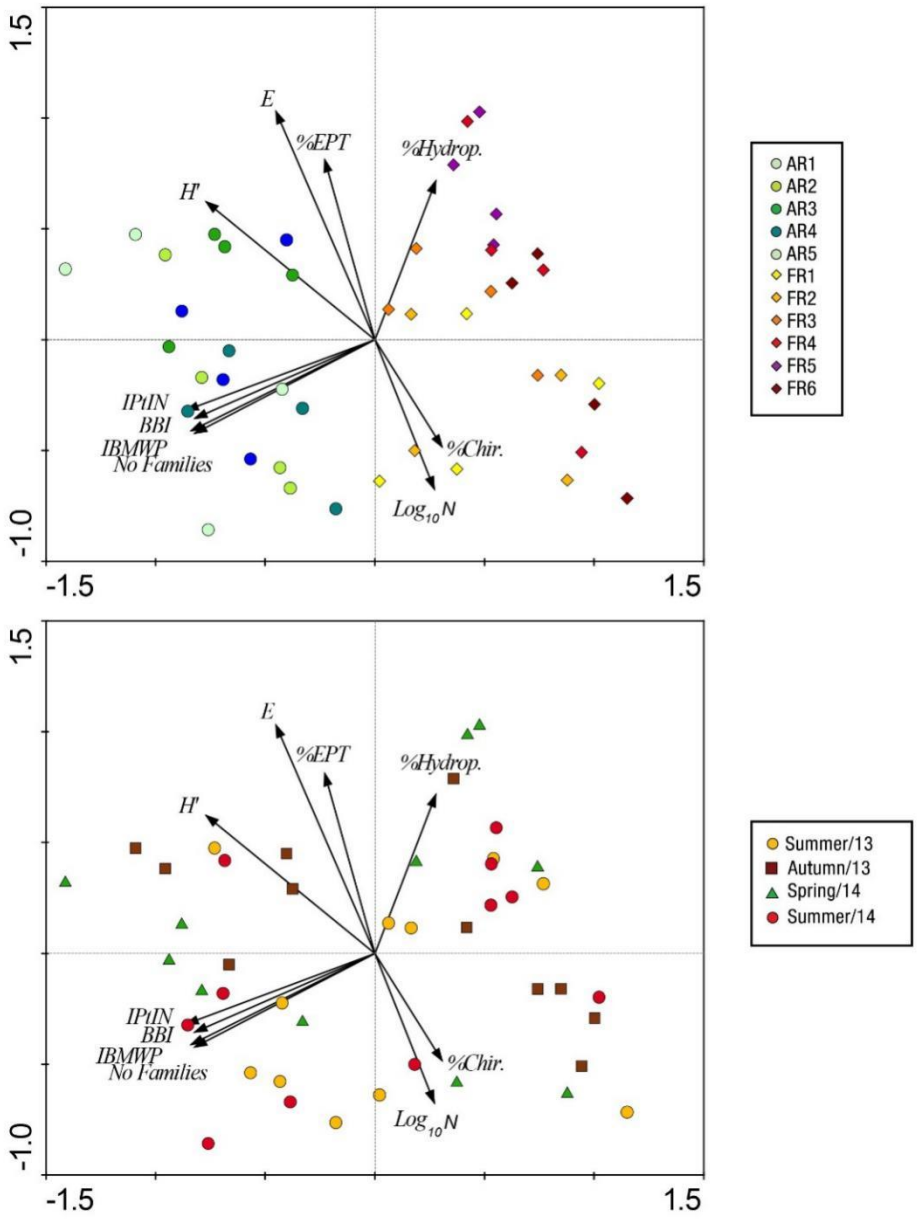

Fig. 7. PCA of the total number of individuals ($Log_{10}N$, being N the total No of individuals) and measures of richness (No families), composition (percent of Ephemeroptera, Plecoptera and Trichoptera orders, % EPT; % Hydropsychidae, % Hydrop.; % Chironomidae, % Chir.), diversity (Pielou's equitability index, E; Shannon-Wiener index, H') and biotic indices (Biotic Belgian Index, BBI; Iberian Biological Monitoring Working Party Index, IBMWP) and North Invertebrate Portuguese index ($IPtI_N$) seasonally determined in the Âncora (AR) and Ferreira (FR) rivers, with samples marked according to (A) sampling sites (AR1 to AR5; FR1 to FR6) and (B) seasons.

The BBI index indicated that AR sites were unpolluted or slightly polluted (classes I or II) and the FR sites were unpolluted to moderately polluted (classes I to III) (Table 8). The IBMWP index indicated that AR sites had good or acceptable quality (class I or II), while FR sites had good to critical quality (class I to IV) (Table 8). According to the $IPtI_N$, almost all AR sites presented high (class I) or good (class II) ecological status in all seasons (except AR3 in summer and autumn of 2013, AR4 in summer of 2013 and spring of 2014 and AR5 sites in autumn of 2013, with moderate ecological status, class III) (Table 8). Most FR sites showed moderate or poor ecological status (except RF1 in summer of 2014, with bad ecological status). The downstream sites, FR4 to FR6, had poor ecological status in all seasons (Table 8).

The horizontal axis of the PCA of the total number of macroinvertebrate individuals (Log_{10} N) and measures of richness, composition, diversity and biotic and $IPtI_N$ indices discriminated between rivers (i.e. AR from FR), explaining 47.4% of the total variability (Fig. 7A). The Shannon-Wiener (H'), biotic (BBI and IBMWP) and multimetric ($IPtI_N$) indices were the parameters that contributed most to this discrimination (Fig. 7A). The vertical axis discriminated between summer and spring samples and autumn samples, explaining 23.0% of the total variability; the total number of organisms, the equitability index (E) and composition measures (% EPT, % Hydropsychidae and % Chironomidae) were the parameters that contributed most to this seasonal pattern (Fig. 7B).

3.4.4. Macrophytes

No significant seasonal or spatial differences were found for the Macrophyte Biological Index for Rivers (IBMR) in both rivers. According to this index, all AR sites presented high ecological status in all seasons (Table 9, Fig. 5). The majority of the indicator species of angiosperms (*Eleogiton fluitans*, *Juncus bulbosus*, *Potamogeton polygonifolius*, *Ranunculus ololeucos*) and bryophytes (*Aneura pinguis*, *Fissidens polyphyllus*, *Fontinalis squamosa*, *Hyocomium armoricum*, *Nardia compressa*, *Racomitrium aciculare*, *Scapania undulata*, *Sphagnum auriculatum*) found in this river are characteristic of environments with low productivity and are mainly found in mountain rivers. However, species of angiosperms moderately tolerant to nutrient disturbance (*Apium nodiflorum*, *Callitriche stagnalis*, *Oenanthe crocata*) and eutrophic indicator species (*Polygonum hydropiper*) were also found.

Table 9.

Scores and corresponding ecological quality classes of the Macrophyte Biological Index for Rivers (IBMR) determined at the sampling sites of the Âncora (AR1 to AR5) and Ferreira (FR1 to FR6) rivers in different seasons. The total number of macrophyte indicator species (No indicator species) identified during the inventory for the IBMR determination, is also given.

Season/ Year	Site	IBMR	
		Score	No indicator species
Autumn/13	AR1	1.43 (I)	10
	AR2	1.46 (I)	11
	AR3	1.33 (I)	7
	AR4	1.48 (I)	11
	AR5	1.33 (I)	7
Spring/14	AR1	1.43 (I)	10
	AR2	1.44 (I)	11
	AR3	1.26 (I)	7
	AR4	1.44 (I)	12
	AR5	1.27 (I)	10
Summer/14	AR1	1.43 (I)	10
	AR2	1.40 (I)	11
	AR3	1.26 (I)	8
	AR4	1.42 (I)	15
	AR5	1.33 (I)	9
Autumn/13	FR1	0.90 (II)	7
	FR2	0.80 (II)	5
	FR3	0.79 (II)	7
	FR4	0.80 (II)	8
	FR5	0.83 (II)	5
	FR6	0.66 (III)	3
Spring/14	FR1	0.74 (II)	9
	FR2	0.80 (II)	11
	FR3	0.83 (II)	15
	FR4	0.78 (II)	12
	FR5	0.68 (III)	8
	FR6	0.66 (III)	4
Summer/14	FR1	0.87 (II)	10
	FR2	0.72 (II)	8
	FR3	0.71 (II)	10
	FR4	0.69 (II)	9
	FR5	0.70 (II)	7
	FR6	0.13 (V)	2

Note: *IBMR ecological quality classes for "small sized streams of North of Portugal"*: score ≥ 0.92, class I (high ecological status); score: 0.69-0.91, class II (good ecological status); score 0.46-0.68, class III (moderate ecological status); score 0.23-0.45, class IV (poor ecological status); score ≤ 0.23, class V (bad ecological status).

The FR presented good ecological status according to the IBMR, with exception of the FR5 and FR6 sites in spring, which showed a moderate ecological status (Table 9, Fig. 5). In autumn and summer, although FR6 showed a moderate and poor ecological status, respectively, there were not sufficient indicator species to produce a reliable index result (less than 4 indicator species found – no confidence in results). In the FR, angiosperm indicator species for moderate pollution (*Callitriche stagnalis*, *Apium nodiflorum*, *Phalaris arundinacea*, *Sparganium erectum* subsp. *neglectum*) were the

most abundant but angiosperm indicator species for eutrophication (*Alisma plantago-aquatica*, *Polygonum hydropiper*, *Typha latifolia*) and bryophytes (*Amblystegium riparium*, *Octodiceras fontanum*, *Fontinalis antipyretica*) indicative of eutrophication were also found.

3.5. Discussion

3.5.1. Environmental quality elements

Physico-chemical parameters in the Ferreira River (FR) varied both seasonally and spatially, whereas in the Âncora River (AR) the fluctuations in these parameters were mostly season-dependent and mainly regulated by factors such as temperature and flow velocity. The good oxygenation found in both rivers was due to the presence of dams, rapids or other areas of turbulence in these watercourses, combined, in the winter, with lower water temperature and higher discharge and flow velocity. COD values suggested a low amount of oxidizable organic material in both rivers.

The lower pH values found in the winter were related to a decrease in primary productivity (decrease in the quantity of carbonic acid in solution); the lower conductivity and TDS values can result from dilution caused by increased discharge. The highest true colour value observed at FR5 can be related to higher nutrient concentrations (also observed in other downstream sites) and to the dissolved iron flowing from an upstream nearby tributary (Teixeira et al. 2014); the precipitation of dissolved iron ions may have contributed to an increased apparent colour.

Some AR sites presented nutrient disturbance, especially in spring and summer, coincident with increased application of fertilizers close to the river banks and lower river discharge. Higher levels of phosphorus were also observed in the winter due to agricultural runoffs caused by the increased precipitation. It is known that agricultural runoffs from fertilized land contribute significantly to the degradation of aquatic ecosystems and have deleterious effects on water quality and on the habitats of river systems (Stone et al. 2005). Moreover, these runoffs vary annually and from basin to basin (Udawatta et al. 2002) and eutrophic rivers, such as the FR, have much slower nutrient uptake rates than oligotrophic rivers (Paul and Meyer 2001).

The high nutrient contents observed in the FR suggest a continuous nutrient input to the watercourse, especially in the spring and summer (Table 5). The downstream sites

(FR4 to FR6) were the most impacted, since they receive diffuse pollution from agricultural land, urban sewage and discharges from Wastewater Treatment Plants (WWTPs). The hydrographic basin of the FR has been subjected to dramatic changes in land use and plant cover, severing the connections between biotic and abiotic natural elements. Besides, the existing WWTPs have already reached their maximum treatment capacity, both in terms of hydraulic level and organic load. Thus, implementation of measures to improve the coverage of the sanitation network and reinforcement/increase of the treatment's efficiency are necessary (Monteiro et al. 2005; PGRH3 2016). The lower nitrate content at FR4 (site with the greatest macrophyte cover), relative to FR5 and FR6, may be due to a higher uptake of nitrate by vegetation. Although nitrate is the primary form of nitrogen used by plants, in excess, it can lead to watercourses being clogged by fast growing macrophytes (EPA 2012).

The vegetation of both studied rivers was found quite altered, mainly in terms of the floristic composition of the riparian communities. In Northwest Portuguese rivers vegetation is usually composed of different types of plant communities, namely a riparian forest, a forbs fringe community and a hydrophilous community dominated by aquatic plants. The riparian forest is usually dominated by alders (*Alnus glutinosa*) accompanied by ash (*Fraxinus angustifolia*) and black willow (*Salix atrocinerea*) (Castro 1997). Indeed, the presence of alders was not recorded in AR1 and the tree layer was completely dominated by several woody alien invasive species (*Acacia* spp.), also observed in the remaining sampling sites. Herbaceous hygrophytes such as *Eupatorium cannabinum*, *Picris hieracioides* and *Cirsium palustre*, typical of forbs fringe communities, were also absent in some sampling sites where they were replaced by nitrophylous communities. Most FR sites showed low riparian cover and low cover of *Carex elata* spp. *reuterana*, pointing to the need for a structural restoration by increasing riparian width and restoring the longitudinal connectivity of riparian woods (Aguiar et al. 2011b). Moreover, a high proportion and cover of alien species were detected in the riparian zone of both rivers, including at sites with high ecological quality (e.g. all class I AR sites), indicating the need to restore the composition of the riparian communities by removing or lowering the cover of alien species and fostering the native species communities (Aguiar et al. 2011b). AR3 in particular, and despite being the only site with channel and river banks in natural condition (HQA and HMS with high ecological quality), showed the greatest coverage of alien species, possibly resulting from the occurrence of forest fires (close to the waterline) in 2012, creating conditions for further dispersion of alien invasive species. Except for FR4, FR sites are located in peri-urban areas with intensive agriculture and had high richness of alien invasive taxa. Some invasive taxa not included in the national lists of invasive species (Decree-Law No. 565/99 of 21 December) were recorded in the

field surveys of the AR (*Crocosmia* x *crocosmiiflora*), the FR (*Amaranthus blitum*, *Amaranthus hybridus*, *Amaranthus powellii*, *Aster* x *salignus*, *Cardamine occulta*, *Cyperus esculentus*, *Cyperus eragrostis*, *Helianthus tuberosus*, *Juncus tenuis*, *Oenothera biennis*, *Oenothera glazioviana*, *Polygonum pensylvanicum*, *Solanum chenopodioides*, *Soliva sessilis*, *Vitis* x *instabilis*, *Vitis labrusca*) and in both rivers (*Conyza bilbaoana*, *Conyza sumatrensis*, *Tradescantia fluminensis*, *Vitis* × *novae-angliae*). It should also be referred that some invasive taxa found in field surveys (*Cardamine occulta*, *Helianthus tuberosus, Juncus tenuis*, *Vitis* x *instabilis, Vitis* × *novae-angliae*) were only recently reported for the first time in Portugal (Verloove and Alves 2016). Local authorities should be alerted, since some of these species have the potential to cause serious nuisance in the near future. Invasions by alien plants in riparian communities are known to alter the structure and function of riparian habitats and threaten biodiversity (Richardson et al. 2007). Furthermore, alien species can reduce ecosystem services provided by riparian zones, affecting flood patterns, water table levels and soil moisture conditions (Meek et al. 2010; Tickner et al. 2001). In order to point out the most urgent mitigation measures to prevent further invasive population growth and spread, future studies should focus on the pressure-level of invasion in both studied rivers to assess the main causes of degradation.

3.5.2. Biological quality elements

Overall, the quality classes obtained from the biotic indices (BBI and IBMWP) were equal to or higher than those obtained with the multi-metric $IPtI_N$. This happens because biotic indices use only one metric or trait (focused on organism tolerances to organic pollution) to evaluate a stream's health rather than taking into account the combined impacts of multiple stressors (Herman and Nejadashemi 2015).

Ephemeroptera, Plecoptera and Trichoptera orders are particularly sensitive to organic pollution within an ecosystem and can thus be used to identify locally impacted regions (Herman and Nejadashemi 2015). In this study, no differences between rivers were found for the % EPT. However, FR sites presented a low abundance or absence of individuals belonging to the Plecoptera order and a greater contribution of the families Baetidae, Caenidae (Ephemeroptera order) and Hydropsychidae (Trichoptera order) to the % EPT. Caenidae and Baetidae families considered to be in the mid-range for tolerance to most of the environmental stressors, namely sedimentation, nutrient enrichment and organic pollution (Harrington and Born 2000; Menetrey et al. 2008). These organisms are swimmers (Bio et al. 2011) and are commonly observed in all

seasons and sites. Though they can be drawn by the current especially during the autumn (higher flow velocity), they can also move easily to local marginal zones where the current is less intense. A predominance of the family Hydropsychidae (mid-range tolerance) is also a recognised indicator of organic pollution. Species belonging to the genus *Hydropsyche* (genus found in AR and FR) are mostly filter-feeders (75%) feeding mostly on living macrophytes and microinvertebrates (Tachet et al. 2002). They are also well adapted to the flow conditions existing in the FR, because of the hooks on their back, which allow them to resist flow entrainment (Tachet et al. 2002).

Organic pollution and the alterations found in the AR riparian corridor did not affect the balance of the macroinvertebrate community, since most sampling sites showed a good or high ecological status according to the $IPtI_N$. In the FR, the macroinvertebrate community showed a strong response to anthropogenic disturbances (e.g. organic pollution, hydromorphological alterations) with the majority of sampling sites having a moderate or poor ecological status. $IPtI_N$ was lower in downstream sites than in upstream ones, especially at FR6. This site, besides having high nutrient content and very bad habitat quality, also had a substrate dominated by sand, which has frequently been referred to as a poor substrate in terms of diversity (Kikuchi and Uieda 2005).

The disappearance of lentic families belonging to the Odonata (e.g. Calopterygidae, Platycnemididae, Lestidae), Hemiptera (Guerridae, Hydrometridae) and Coleoptera (Dytiscidae) orders was reflected in a decline of the ecological status from summer to autumn 2013, at AR5 (good to moderate). Hydrological processes affect instream organisms both directly, by applying hydrodynamic forces of varying magnitude (Giller and Malmqvist 1998), and indirectly, by determining substrate composition, water chemistry and habitat availability (Hart and Finelli 1999). Nevertheless, the structural characteristics of these sites allow a rapid recolonization of the whole system. This leads us to believe that the populations are relatively stable; that is, they are already adapted to flow fluctuations and adopted survival strategies. A diversified and relatively stable substrate, with interstitial spaces providing important refuges for recolonization, contributes to this stability. The decrease in ecological status from summer to autumn 2013 at FR1, FR2 and FR3 (moderate to poor) was also due to disappearance of lentic taxa, namely, families that are moderately tolerant to organic pollution belonging to Coleoptera (e.g. Dytiscidae, Gyrinidae) and Hemiptera (e.g. Gerridae, Nepidae) orders, and sensitive families belonging to Odonata (Gomphidae), Trichoptera (e.g. Leptoceridae, Limnephilidae), Ephemeroptera (e.g. Leptophlebiidae, Ephemerellidae) and Plecoptera (Leuctridae) orders. In autumn, the increase in the % Oligochaeta in both rivers seems to be mainly related to the increase of hydrological parameters, since they live buried in the sediment thus resisting entrainment and feeding on particulate organic

matter (Tachet et al. 2002). Bouckaert and Davis (1998) associated a greater number of Oligochaeta to a greater deposition of particulate organic matter, emphasizing that macroinvertebrates' communities can be more influenced by flows of dissolved gases and by particulate organic matter than directly by flow velocity and other forces of entrainment. In the present study, higher percentages of organic matter in sediment were also observed in the AR, during the autumn. In the spring and summer of 2014, the number of individuals belonging to the Gastropoda class (Mollusca) increased in the FR, with predominance of families tolerant to organic pollution (e.g. Physidae; Alba-Tercedor and Sánchez-Ortega 1998). Munné and Pratt (2011) analysed the differences in composition and structure of macroinvertebrate communities in five Mediterranean rivers and reported that macroinvertebrate communities varied significantly between hydrological conditions. Dry and wet periods differ more (within each season) than seasons as such (spring vs summer). In most papers, the hydrological period is mainly considered in terms of season (i.e. spring vs summer) and not hydrology, which can vary greatly within a season and between years, depending on climate conditions. FR1 and FR3 sites presented higher ecological status in the summer 2013 compared to summer 2014. This seemed to be related to both a higher number of riffles in the summer 2013, which creates better conditions for the colonization of more sensitive and flow requiring taxa (e.g. Ephemeroptera, Plecoptera and Trichoptera), and the channel cleaning actions that took place at these sites during the summer of 2014. These actions included the removal of macrophytes at FR1 and the removal of macrophytes, woody debris, and stones at FR3 (recreational area), which decreased physical habitat heterogeneity and consequently reduced refuge opportunities for aquatic organisms and ultimately the density and diversity of benthic macroinvertebrates.

The anthropic pressures on both rivers, especially in summer (increased nutrient content) do not seem to have caused an imbalance in the macrophyte community according to the IBMR index. AR sites presented high ecological status and most sampling sites of the FR presented good ecological status. In spring, downstream sites FR5 and FR6 had high nutrient concentrations and moderate ecological status, while FR4 showed good ecological status, which was due to a seasonal island (submerged during winter) where a large community of macrophytes developed, including indicator species. The IBMR, as most macrophyte trophic indices requires a certain number or biomass of indicator taxa to be present at a river site in order to ensure a reliable indication. For example, at FR6 in the autumn and summer 2014, there were not sufficient indicator species (i.e. less than 4 indicator species) to produce a reliable result (Aguiar et al. 2014a). Neglecting important indicator taxa a reliable indication of river trophic status may often be impossible due to the presence of too few indicator taxa.

Thus, an adjustment of the indicator species lists to local conditions is highly recommended (Schneider 2007). Regarding the IBMR index and in order to improve its robustness, we suggest the inclusion of two species characteristic of oligotrophic environments, which were recorded in the AR and have often been recorded in most of the fluvial systems of mainland Portugal, namely *Juncus heterophyllus* and *Baldellia alpestris*; the latter is endemic to the mountains of northern Portugal and northwest Spain (Kozlowski and Matthies 2009; Romero and Onaindia 1995).

The ecological classification obtained with the $IPtI_N$ was lower than that obtained with the IBMR for both rivers, suggesting that macroinvertebrates are more sensitive to anthropogenic pressures (eutrophication/organic pollution, hydromorphological degradation) than macrophytes. This result is in agreement with previous studies, which stated that different BQEs/metrics are impacted differently by anthropogenic pressures (e.g. Hering et al. 2006a; Johnson et al. 2006b; Johnson and Hering 2009; Marzin et al. 2012). The $IPtI_N$ multimetric index is mainly suitable for detecting multiple pressure effects such as organic pollution, acidification, hydrological and morphological alterations and general degradation in rivers (INAG 2009). Although the effects of eutrophication and organic pollution (e.g. increased BOD) are of different origin, they are often correlated. Thus, macroinvertebrates can in most cases be used to detect both types of stressors (Hering et al. 2006b). The IBMR index was originally developed to detect organic pollution and to characterise and monitor the trophic state of surface watercourses (Aguiar et al. 2014a). In the present study, the evaluation of the ecological status of the studied rivers using only macrophyte responses to nutrient enrichment provided a partial evaluation of the effects of the stressors affecting the integrity of the river ecosystems.

Besides water quality parameters, the evaluation of the responses of other disturbance variables, such as hydrological and physical disturbances, is particularly important for Mediterranean rivers, given the water constraints and the long-term agricultural use of river surroundings (Aguiar et al. 2011a). Next to the analysis of the presence and coverage of indicator species, the analysis of the structural and functional components of the riparian ecosystem though the RVI index, greatly contributed to the assessment and interpretation of the ecological status of both rivers (holistic understanding of the ecosystems). This index may give ecological support for future management and planning decisions, reducing management and restoration planning costs (Aguiar et al. 2011b), which justified the need and effort to carry out an inventory of aquatic and bankside plant species at each sampling site. The RVI has been referred as being an effective method for bioassessment in Mediterranean rivers (Aguiar 2009; Aguiar et al. 2011a, b; Aguiar et al. 2014b). However, other indices can be used to assess

local features of vegetation for other regions (e.g. riparian quality index, González-del-Tánago and García-Jalón 2006; plant index of biotic integrity, Simon et al. 2001; Rothrock et al. 2008).

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04

CHAPTER

Assessing environmental status of fluvial systems employing a macroinvertebrate multi-taxa and multi-biomarker approach

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Journal-Article

Rodrigues C, Bio A, Guimarães L, Fernandes VC, Delerue-Matos C, Vieira MN. Assessing environmental status of fluvial systems employing a macroinvertebrate multi-taxa and multi-biomarker approach. (submitted for publication) – based on Chapter 4

Conference-poster

Rodrigues C, Fernandes VC, Cristina Delerue-Matos C, Vieira MN (2017) Occurrence of Organophosphorus pesticide in sediments from Portuguese rivers. 10º Encontro Nacional de Cromatografia (ENC 2017), Bragança, Portugal, December 4-6.

Assessing the environmental status of fluvial ecosystems employing a macroinvertebrate multi-taxa and multi-biomarker approach

4.1 Abstract

Biomarkers have been proposed as sensitive early-warning tools for biological effects in aquatic organisms. In this context, the main aim of this study was to investigate the potential usefulness of a battery of biomarkers evaluated in different benthic macroinvertebrate taxa to discriminate aquatic ecosystems with different levels of ecological quality and to provide further clues supporting environmental management.

The study took place during the autumn of 2013 and the spring and summer of 2014, and the study cases were two Northern Iberian rivers (Âncora and Ferreira rivers), differing in their ecological status.

The biomarkers determined are widely recognized, comprising a large set of biochemical responses: activity of enzymes cholinesterases (ChE), glutathione *S*-transferases (GST), catalase (CAT) and lactate dehydrogenase (LDH), and the levels of lipid peroxidation (LPO). They were assessed seasonally and in different macroinvertebrate taxa. Thirteen water physico-chemical parameters were also seasonally determined and the concentration of seven organophosphorus pesticides and the percentage of thirty-two trace metals in sediments were determined in the spring.

This is particularly useful for water authorities, to take actions against further deterioration of the ecological status. Multivariate analyses showed distinct patterns of biological response for the *Calopteryx* spp., Chironomidae and *Baetis* spp. taxa. *Calopteryx* spp. and Chironomidae, in particular, showed distinct response patterns for the two rivers, which were fairly stable across seasons. This study sets the foundations for future cost-effective biomonitoring campaigns in Mediterranean rivers, allowing to establish historical data important to understand ecosystem evolution, as well as baseline levels of diagnostic biomarkers in informative macroinvertebrate taxa. The latter not only fulfil WFD regulatory requirements but also foster efficient detection of pollution incidents threatening ecosystems health.

Keywords: Biochemical biomarkers; Macroinvertebrates; Water Framework Directive; Integrated monitoring; Neurotoxicity; Biotransformation; Oxidative stress

4.2. Introduction

In 2000, the EU Water Framework Directive (WFD; EC 2000) was established as the main legal instrument enforcing management, protection and restoration of aquatic ecosystems. It conceptually changed water management in all EU Member-States by using ecosystem health and sustainability principles as the basis for decisions. Biological Quality Elements (BQEs), which integrate the effects of all stressors, are now used to assess the ecological status of surface waters. However, ecosystems are diverse, complex and fluctuating entities. For this reason the development of adequate ecological assessment and classification systems has been one of the most technically challenging aspects faced in the application of the WFD (EU 2003).

For the assessment of fluvial ecosystems, indices based on the analysis of fish, phytobenthos, macrophytes and benthic macroinvertebrates are the BQEs recommend by the WFD. Each of these indices relies on surveys of the community structure, for the respective BQEs, to evaluate and classify rivers' ecological quality. They detect relevant effects that usually cause the elimination of one or several species from a particular site (Damásio et al. 2011). While having high ecological relevance, because of the loss of biodiversity these indices are of limited interest to anticipate specific protection measures required to maintain ecological quality or prevent its damage. The need for rapid and sensitive tools to reveal sub-lethal effects in aquatic organisms, able to anticipate future detrimental ecological effects, has raised interest in biomarkers, as useful tools to complement the information from community structure indices. Measured at lower levels of biological organisation, biomarker responses occur on shorter timescales. In particular, multi-biomarker evaluations can reveal early signs of exposure and adverse outcomes, translating the integrated impact of natural stressors and chemical contaminants to which animals are exposed (Allan et al. 2006; Hagger et al. 2006). The inclusion of macroinvertebrate biomarkers in the biomonitoring of contamination in rivers and streams has thus been increasingly reported (e.g. Berra et al. 2004; Bonzini et al. 2008; De Castro-Català et al. 2015; Minutoli et al. 2013; Olsen et al. 2001). However, studies aiming to evaluate the ecological quality of rivers using both biomarker- and community-based approaches in macroinvertebrates are still rare (Barata et al. 2005; Damásio et al. 2011; Prat et al. 2013; Puértolas et al. 2010). And most of these studies

were based on the determination of biomarkers from a single and tolerant macroinvertebrate species. Some authors, however, have found that the evaluation using a single species may result in either under or over estimation of the risk, depending on the species selected (Berra et al. 2004; Bonzini et al. 2008). These authors, determined biochemical biomarkers in different macroinvertebrate taxonomic groups, mainly in families of the Diptera, Plecoptera, Odonata, Ephemeroptera, Trichoptera, Amphipoda and Isopoda orders (Berra et al. 2004; Bonzini et al. 2008), rather than in one single species. Their studies report differential taxa sensitivities for several biomarkers (e.g. involved in neurotransmission, biotransformation and antioxidant defences) (Berra et al. 2008; Bonzini et al. 2008), suggesting that particular attention should be payed to the selection of the taxa employed in a given monitoring scheme, so as to avoid biased risk estimations. Though samples obtained for higher taxonomic levels than species (genus, families) may also include species with some differential sensitivity, the above mentioned results suggest that this approach of using higher levels of taxonomic resolution may be effective in detecting subtle gradients of toxic substances and their effects on the exposed biota. Moreover, from the phylogenetic perspective, sister species (e.g. species in the same genus) are likely to show similar physiological responses (Colin et al. 2016). Therefore, further studies including multiple biomarkers, environmental stressors, macroinvertebrate taxa and river types are necessary to evaluate the wider usefulness of this approach and contribute to establish efficient and cost-effective biomarker strategies, indicative of future ecological damage. In this context, the aims of this study were: i) to investigate if a battery of biomarkers evaluated in different benthic macroinvertebrate taxa could discriminate aquatic ecosystems with different levels of ecological quality; ii) to understand if biomarker data can help identifying potential problems or sources of contamination affecting aquatic biota, complementing the information given by ecological quality indices, and iii) to identify the most favourable taxa and season(s) to be used in a multi-biomarker and multi-taxa analysis in cost-effective biomonitoring programmes. To tackle these objectives a seasonal study was carried out in two Northern Iberian rivers with different ecological status. Thirteen physico-chemical water parameters were measured in both rivers. The concentrations of seven organophosphorus pesticides and the percentages of thirty-two trace metals in the sediment were also determined in the spring (season of major application of pesticides in agriculture). A battery of biomarkers of neurotoxicity (cholinesterases activity), biotransformation (glutathione-S-transferases), antioxidant defences (catalase), oxidative damage (lipid peroxidation) and energy metabolism (lactate dehydrogenase) was seasonally assessed in different macroinvertebrate taxa collected at various sites in the two rivers.

4.3. Materials and methods

4.3.1. Study area and sampling sites

This study was conducted during the autumn of 2013 and spring and summer of 2014, in two Northern Portuguese rivers integrated in the Natura 2000 Network; Âncora River (41°48'5.63"N, 8°46'28.57"W) and Ferreira River (N41°11'15.06", W8°27'25.47") (Fig. 8A). These areas differ in their ecological status, as previously determined in Chapter 3 through biological and hydromorphological indices recommended by the WFD. Geographically, the Âncora River (AR; Fig. 8B) springs from Serra de Arga, in the Viana do Castelo municipality and runs approximately 17.91 km through a steep bedrock, before flowing directly into the Atlantic Ocean, in the Caminha municipality (PGRH1 2016). The Ferreira River (FR; Fig. 8C) springs in the Paços de Ferreira municipality, has an approximate length of 22.30 km, and joins the Sousa River in the Gondomar municipality (Monteiro et al. 2005; PGRH3 2016).

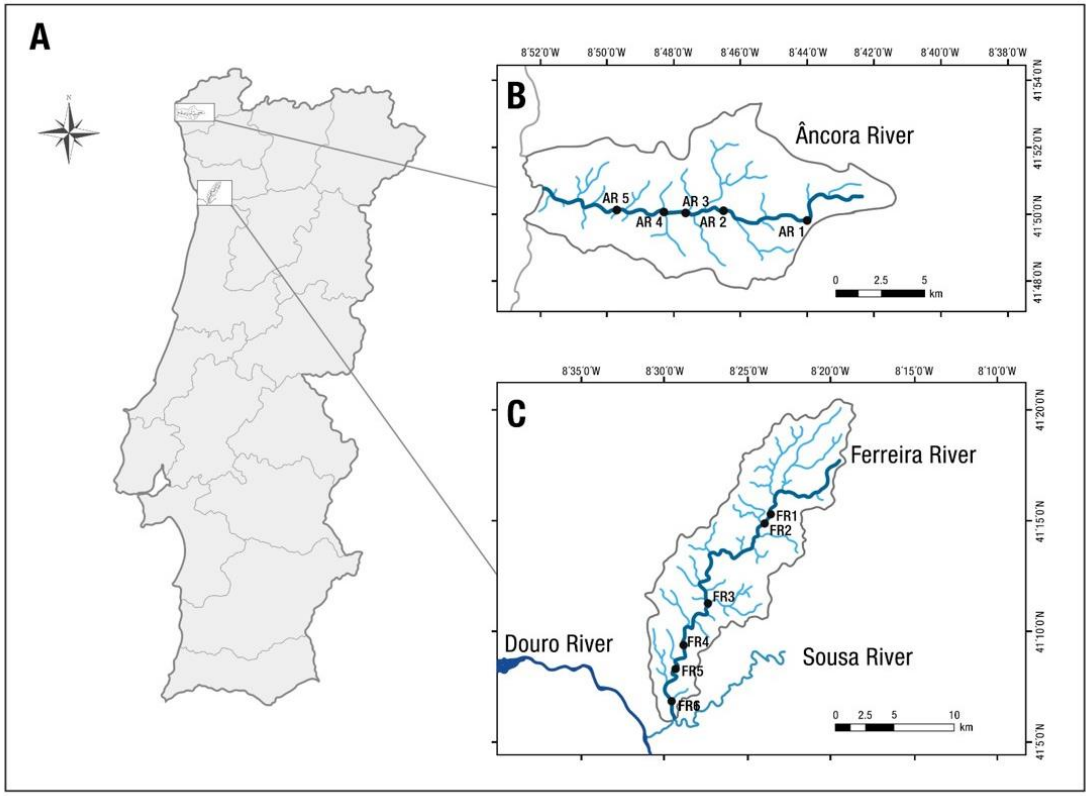

Fig. 8. Geographical situation. (A) Location of the hydrographic basins of River Âncora, B, and River Ferreira, C (rectangles), in Portugal mainland; (B) Map of the hydrographic basin of the Âncora River and the distribution of the sampling sites in the River Âncora (AR1 to AR5); (C) Map of the hydrographic basin of the Ferreira River and the distribution of the sampling sites in the River Ferreira (FR1 to FR6).

Both the AR and FR belong to the "small sized streams of the North" national river type (catchment area < 100 km^2), which reflects the country's northern climate with high annual average precipitation (mean: 1190.25 mm ± 357.80) and low annual average temperature (mean: 12.42 °C ± 1.26) (INAG 2008). Both rivers are mainly located in areas with siliceous geology (schist, granite), presenting low mineralization (INAG 2008). In the hydrographic basin of the AR, the main sector of activity is agriculture (PGRH1 2012) and in the hydrographic basin of the FR, the industrial activity is largely dominant, followed by agriculture (Monteiro et al. 2005).

All the AR sampling sites (AR1 to AR5; Fig. 6B) are integrated into the Natura 2000 Network (PTCON0039, Site "Serra de Arga"); one of the FR sampling sites (FR4, Fig. 8C), is also included in the Natura 2000 Network (PTCON0024, Site "Valongo").

4.3.2. Physico-chemical, hydromorphological and biological parameters

Water physico-chemical parameters were seasonally monitored (autumn of 2013 and spring and summer of 2014, i.e., except in winter due to high river discharge during this season), simultaneously with the macroinvertebrate sampling campaigns. Water temperature (°C), pH, dissolved oxygen concentration (mg O_2/L) and percent of saturation (% DO), total dissolved solids (TSS, mg/L), conductivity (µS /cm) and salinity (PSU) were measured using a multiparameter portable meter (HI 9829*,* Hanna Instruments). Chemical oxygen demand (COD, mg/L), nutrient concentrations (nitrates, nitrites, ammonium ion and total phosphorus in mg/L) and total suspended solids (mg/L) were determined in the laboratory. COD and nutrients were determined using a Hanna Instruments multiparameter bench photometer following the manufacturer's protocols (Hanna Instruments 2014) and TSS were determined according to the method described by the American Public Health Association (APHA 1992). Physico-chemical parameters were classified considering the maximum limit values established for the "good" ecological status in Northern Portuguese rivers (INAG 2009).

The scores of the biological and hydromorphological indices, determined for the same sites and seasons in which organisms for biomarker analysis were collected. (obtained from Chapter 3) were used for statistical tests and multivariate analysis. Biological quality of sampling sites was assessed through the North Invertebrate Portuguese Index based on the benthic macroinvertebrate community ($IPtI_N$; INAG 2009; EC 2013). The hydromorphological quality was assessed through the Habitat Quality Assessment index (HQA) and the Habitat Modification Score (HMS) index, following the

River Habitat Survey methodology (RHS; Raven et al. 1997, 2002, 2009). The HQA index provides an indication of the overall habitat diversity in the river's channel and corridor, and the HMS index provides an indication of artificial modifications in the river's channel morphology.

4.3.3. Sediment analysis

Sediment sampling campaigns were carried out in late spring, after a month without rainfall events to avoid their influence on the results (e.g. runoff, dilution or infiltration).

4.3.3.1. Pesticides analysis

Seven organophosphorus pesticides used in agriculture in the studied areas were analysed, namely dimethoate, diazinon, chloropyrifos-methyl, parathion-methyl, malathion, chlorpyrifos and chlorfenvinphos. Sediment samples for pesticide analysis were collected (in triplicate) at each sampling site from the top layer (1 cm deep), as suggested by Rubal et al. (2009), and kept at −20 °C until analysis.

4.3.3.1.1. Reagents

Standards of 7 organophosphorus pesticides (dimethoate, diazinon, chloropyrifos-methyl, parathion-methyl, malathion, chlorpyrifos and chlorfenvinphos) and the internal standard (IS) ─ triphenyl phosphate (TPP) ─ were obtained from Sigma-Aldrich (St. Louis, MO, USA). All standards were of ≥ 99% purity and all solvents were of chromatography grade. Acetonitrile (MeCN) and n-hexane were from Merck™ (Darmstadt, Germany).

QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) tubes containing 6 g of magnesium sulphate ($MgSO_4$), 1.5 g of sodium chloride (NaCl), 0.750 g of disodium citrate sesquihydrate ($Na_2HCit\ 1.5H_2O$) and 1.5 g of sodium citrate dehydrate ($Na_3Cit\ 2H_2O$) and the clean-up tube containing 50 mg of PSA, 150 mg of magnesium sulphate ($MgSO_4$) and 50 mg of C18, were purchased from UCT® (Bristol, PA, USA). Ultrapure water of 18.2 MΩ-cm came from a Simplicity 185 apparatus (Millipore from Molsheim, France).

Stock solutions of each pesticide were prepared at 10000 µg/L concentrations in n-hexane and stored at −18 °C. Working standard mixture solutions of the different desired concentrations were prepared in n-hexane and were used as spiking, calibration,

and control solutions. For matrix-matched curves, six solutions (between 20 and 300 ng/g of dry weight) and three spiking levels (50, 100, 200 ng/g d.w.) were prepared. The IS solution was added in all experiments to have a final concentration of 100 ng/g d.w..

4.3.3.1.2. Extraction procedure

After lyophilisation sediment samples were sieved using a sieve of 2 mm of diameter (i.e., includes granule, sand, silt and clay, representing these sediment size classes more than 50% of the sediment at all sampling sites; Chapter 3) . Material passing through a 2 mm sieve were ground in a grinder (Ultra Centrifugal Mill ZM 200) passed through a 0.25 mm sieve to obtain a homogeneous sample, before being extracted and analysed. The extraction procedure was carried out according to the method of Fernandes et al. (2013). A 5 g portion of each sediment sample was weighed into a 50 mL polypropylene (PP) centrifuge tube. For validation experiments, spikes of 50 µL, 100 µL and 200 µL using appropriately concentrated solution were made to yield the desired analyte concentrations in sediment samples. Except for samples needed for matrix-matched calibration standards, a 10 µL IS solution in n-hexane was added to all samples and reagent blanks (to yield 100 ng/g d.w.). An aliquot of the upper layer in each tube was transferred to a vial and concentrated just to dryness, using a gentle stream of nitrogen. Residue was reconstituted in n-hexane. Finally, the sample was capped, vortexed and placed for GC (Gas Chromatography) analysis.

4.3.3.1.3. Analysis by Gas Chromatography – Flame Photometric Detector (GC-FPD)

Pesticides were analysed using a Shimadzu GC–2010 with a flame photometric detector (FPD) with phosphorus filter. The separation was achieved on a capillary column with 30 m, ZB-XLB (0.25 mm internal diameter, 0.25 µm film thickness, Zebron, Phenomenex). The GC oven temperature program was optimized to separate the organophosphorus pesticides as follows: 50 °C and held for 1 min, ramped at 10 °C/min to 140 °C and held for 1 min, ramped at 5 °C/min to 180 ºC and held for 2 min, and finally ramped at 5 °C/min to 270 ºC, at which it was held for 5 min. The FPD port was at 250 °C splitless mode, and the detection was carried out at 290 °C. Helium (Linde Sogás) was used as carrier gas at constant flow rate of 1 mL/min. The system was operated by GC-Solution Shimadzu software.

4.3.3.2. Determination of sediment trace elements – X-ray fluorescence (XRF)

Sediment samples for trace elements analysis were collected from different points at each sampling site to form a composite sample (of each site). In laboratory, all sediment samples where dried in an oven at 65 °C for about 24 hours. The sediment sample with ≤ 0.25 mm was was used for the analysis of trace elements (required size for X-ray fluorescence analysis). Each sediment composite sample was placed in a standard sample cell from Instru-Med, using a small stainless-steel spatula. A paper disc was holding the soil firmly against the upper Polypropylene X-Ray Film TF-240–255, gauge 4 µm, and 2.5 cm diameter, from Premier Lab-supply, when the sample cup was inverted.

The XRF analyser was a Niton® XL3t, with a detector of high-performance Si PIN diode equipped with a miniature silver anode X-ray tube and an excitation potential of up to 40 kW. The equipment was operated on mining mode. The sample cups were placed in the equipment and the analysis was started from a laptop computer that was directly connected to the XL3t instrument. A qualitative analysis of 32 trace elements (Mo: molybdenum, Zr: zirconium, Sr: strontium, U: uranium, Rb: rubidium, Th: thorium, Pb: lead, As: arsenic, Hg: mercury, Au: gold, Zn: zinc, W: tungsten, Cu: cooper, Ni: nickel, Co: cobalt, Fe: iron, Mn: manganese, Cr: chromium, V: vanadium, Ti: titanium, Sc: scandium, Ca: calcium, K: potassium, S: sulfur, Ba: barium, Cs: cesium, Te: tellurium, Sb: antimony, Sn: tin, Cd: cadmium, Ag: silver, Pd: palladium) was performed. In the hydrographic basin of the AR, the main sector of activity is agriculture and in the hydrographic basin of the FR, the industrial activity (e.g. metallurgical industries, furniture polishing factories, hardware, mechanical locksmithing, slite cut exploitation) is largely dominant followed by agriculture, with a greater number of potential sources of trace metals pollution. XRF was used to measure elements in the sediments and the results were showed in percentage (%).

4.3.4. Biomarkers

4.3.4.1. Macroinvertebrates sampling and storage

Different tolerant and sensitive benthic macroinvertebrate taxa were selected for biomarkers analysis, based on their presence in most sites of both rivers (according to preliminary data obtained in spring of 2013). Organisms were seasonally sampled (autumn, in November 2013; spring, in June 2014; summer, in September 2014) at each sampling site of both the AR and FR (Table 10). For both rivers, despite increased sampling effort, not all taxa could be found in all of the studied seasons.

Table 10.
Benthic macroinvertebrate taxa (genus or family) sampled for biomarkers analysis in the Âncora and the Ferreira rivers in each season.

Seasons	Âncora River	Ferreira River
Autumn	Baetidae (*Baetis* spp.)	Baetidae (*Baetis* spp.)
	Calopterygidae (*Calopteryx* spp.)	Calopterygidae (*Calopteryx* spp.)
	Aeshnidae (*Boyeria* spp.)	Chironomidae
	Gomphidae (*Gomphus* spp.)	
Spring	Baetidae (*Baetis* spp.)	Baetidae (*Baetis* spp.)
	Calopterygidae (*Calopteryx* spp.)	Calopterygidae (*Calopteryx* spp.)
	Aeshnidae (*Boyeria* spp.)	Aeshnidae (*Boyeria* spp.)
	Chironomidae	Chironomidae
	Gomphidae (*Gomphus* spp.)	Hydropsychidae (*Hydropsyche* spp.)
	Leptophlebiidae (*Abrophlebia* spp.)	Nepidae (*Nepa* spp.)
	Ephemereliidae (*Ephemerella* spp.)	Caenidae (*Caenis* spp.)
	Polycentropodidae	Simuliidae
Summer	Baetidae (*Baetis* spp.)	Baetidae (*Baetis* spp.)
	Calopterygidae (*Calopteryx* spp.)	Calopterygidae (*Calopteryx* spp.)
	Aeshnidae (*Boyeria* psp.)	Aeshnidae (*Boyeria* spp.)
	Chironomidae	Chironomidae
	Gomphidae (*Gomphus* spp.)	Hydropsychidae (*Hydropsyche* spp.)
	Leptophlebiidae (*Abrophlebia* spp.)	Nepidae (*Nepa* spp.)
	Ephemereliidae (*Ephemerella* spp.)	Caenidae (*Caenis* spp.)

Sampling of the biological material (with a hand net of 500 µm mesh-size) took place in different organic (macrophytes and coarse particulate organic matter) and inorganic (blocks, stones, gravel and sand + silt + clay) substrates that covered the river bed. Organisms were immediately transported to the laboratory, in plastic containers filled with river water.

Each taxonomic group (Table 10) was sorted out alive, directly from the plastic containers (with tweezers for Odonata and Trichoptera and plastic pipettes with tips of different sizes for Diptera and Ephemeroptera), and morphologically identified to the genus or family level (Tachet et al. 2002) using a magnifying glass. For some taxa, identification to the genus-level was not possible (Simuliidae, Chironomidae and Polycentropodidae families), or would be very time consuming, requiring a microscope to see morphological details, and stressful for the organisms.

Five to one hundred benthic macroinvertebrates of each taxon, depending on the sizes of the organisms (5 each of Trichoptera: *Hydropsyche* spp. and Polycentropodidae; Odonata: *Gomphus* spp., *Boyeria* spp., *Calopteryx* spp.; Hemiptera: *Nepa* spp.; 20 each of Ephemeroptera: *Baetis* spp., *Caenis* spp., *Abrophlebia* spp.and *Ephemerella* spp.; 50 and 100 each of Diptera: Simuliidae and Chironomidae, respectively; organisms of the same taxon were of similar size), were placed in different microtubes which were immediately frozen in liquid nitrogen. Microtubes were stored at −80 ºC until biomarkers analyses. Before this procedure, preliminary laboratory tests were made to optimize the removal of organisms from the containers, so as to minimise stress to organisms, as well as to determine the number of organisms of the same taxa to be integrated in each pool, so as to ensure the amount of biomass needed to record enzymatic activity.

4.3.4.2. Biomarkers analysis

In this study, as well as in the majority of studies using insect larvae (e.g., Barata et al. 2005; Berra et al. 2004; Bonzini et al. 2008; Minutoli et al. 2013), the analysis of all biomarkers was performed in triplicate pools (three independent replicates for each taxon using whole body of the organisms), per sampling site and taxon. For the determination of lactate dehydrogenase (LDH) activity, the content of the microtubes was homogenized in ice-cold Tris/NaCl buffer (pH 7.2, Tris 81.3 mM; NaCl 203.3 mM) and centrifuged at 3300 x *g*, for 3 min at 4 °C after three frozen/unfrozen cycles (at −20 ºC and room temperature). The resulting supernatants were collected and LDH activity was determined by measuring the amount of pyruvate consumed due to NADH oxidation at 340 nm, following the method of Vassault (1983) adapted to microplate (Diamantino et al. 2001). For the determination of acetylcholinesterase (AChE) activity, the content of the microtubes was homogenized in ice-cold phosphate buffer (pH 7.2; 0.1 M) and centrifuged at 3300 x *g*, for 3 min at 4 °C. The resulting supernatants were used to measure AChE activity following the Ellman's method (Ellman et al. 1961), adapted to microplate (Guilhermino et al. 1996). Briefly, ChE determination was performed by

quantifying the hydrolysis of acetylthiocholine by enzymatic action, thus producing ion acetate and thiocholine; the latter product complexes with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), producing a coloured compound. The formation of this compound was followed at 412 nm in a microplate reader during 5 min. Acetylthiocholine was used as substrate in all the assays and no distinction was made between different forms of cholinesterase (ChEs) that might be present. For the determination of glutathione *S*-transferase (GST) and catalase (CAT) activities and lipid peroxidation levels (LPO), the content of the microtubes was homogenized in phosphate buffer (with 0.1% Triton X-100; pH = 7.0; 50 mM) and centrifuged at 15.000 x *g* for 10 min at 4 °C. The supernatants were divided in aliquots, one for each biomarker analysis (GST, CAT and LPO). GST activity was quantified by the conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm each 10 s during 5 min, following the method of Habig et al. (1974) adapted to microplate (Frasco and Guilhermino 2002). CAT activity was determined by measuring the H_2O_2 consumption at 240 nm during 30 s, according to Aebi (1974). The extent of lipid peroxidation (LPO) was measured by the quantification of thiobarbituric acid-reactive substances (TBARS), according to the method described by Buege and Aust (1978). Briefly, after precipitation of proteins with trichloroacetic acid, this methodology is based on the reaction of the lipid peroxidation by-products, such as malondialdehyde (MDA) with 2-thiobarbituric acid (TBA). All enzymatic activities were expressed as nmol/min/mg protein, except for catalase that was expressed as µmol/ min/mg protein. The amount of TBARS was expressed as nmol of MDA equivalents/mg protein. All measurements were performed in triplicate except for LPO which was measured in duplicate. Protein of all samples was quantified according to the method of Bradford (1976), adapted to microplate (Guilhermino et al. 1996), using bovine γ-globulins (Sigma-Adrich, USA) as protein standard and a wavelength of 600 nm. All microplate determinations were carried out in a Thermo Scientific™ Multiskan GO Microplate Spectrophotometer. CAT activities were determined in a UV/VIS spectrophotometer (UV-3100PC, VWR). All enzymatic activities, and respective protein measurements, were done at a constant temperature of 25 °C.

4.3.5. Statistical data analysis

The spatial variation of biomarker responses in the different macroinvertebrate families in each season and river was visualized in Tukey Boxplots, showing data distribution, medians and quartiles (Chambers et al. 1983).

Since most of the variables analysed in this study failed the Shapiro-Wilk normality test and/or the Levene test for homogeneity of variance across groups, we opted for non-parametric statistical testing. Differences between rivers in each season regarding environmental (water physico-chemical parameters, hydromorphological indices and sediment parameters, i.e., percentage of metals and concentration of pesticides), and biological parameters (community index and biomarkers responses in the taxa that were common to both rivers, i.e., Chironomidae, *Baetis* spp., *Calopteryx* spp. and *Boyeria* spp.), were assessed using the Kruskall-Wallis test. Post-hoc pairwise Wilcoxon rank sum tests, corrected for multiple testing, were performed to see which water physico-chemical parameters and hydromorphological and biological indices differed (Hollander and Wolfe 1973) among seasons or among sites within each river. For all statistical tests performed, the significance level was set at 0.05.

Multivariate analysis was used for a better assessment of biomarker applicability for diagnostic purposes. To minimize problems of missing values, high dimensionality, and difficulty in obtaining complete data for some taxa (resulting from their high sensitivity to environmental quality), multivariate analysis was done using only data from the more widespread, abundant or bigger-sized (requiring less organisms for the biomarkers determinations) macroinvertebrate taxa, i.e., *Calopteryx* spp., *Baetis* spp. and *Chironomidae*. Furthermore, multivariate analysis were restricted to spring and summer data, because these were the seasons for which all biomarkers could be determined at all sampling sites in both rivers. First, a detrended correspondence analysis (DCA) was applied to calculate the relative length of the gradient and decide on the appropriate data analysis technique (i.e., the use of unimodal or linear response analyses). Then and since all of our DCA gradient lengths were < 2, a Principal Component Analysis (PCA) was used to identify the main patterns of taxon-related, spatial and seasonal variation in biomarkers responses (according to Ter Braak and Prentice 1988). To assess response patterns of the three taxa, PCA was done first using biomarker levels and the macroinvertebrate index as quantitative variables, taxa and season (spring and summer) as qualitative supplementary variables, and water physico-chemical parameters and hydromorphological indices as supplementary quantitative variables. Following the principal components (PC) extracted and the analysis of the cloud of individuals, subsequent PCAs were carried out for each taxon individually, with river and season as supplementary qualitative variables.

Box-plots, statistical tests and multivariate analyses were performed with the Statistical program R, package version 3.4.2, including the Rcmdr and FactoMineR packages (R Core Team 2017; Fox and Bouchet-Valat 2018; Le et al. 2008).

4.4. Results

4.4.1. Physico-chemical parameters

Significant differences between the Âncora (AR) and Ferreira (FR) rivers ($p < 0.05$) were found for dissolved oxygen (concentration, DO; % saturation, % DO) in summer, ammonium ion in autumn and water temperature, nitrates, nitrites, conductivity, salinity, total dissolved and suspended solids in all seasons (Table 11).

FR reached higher values than AR for most parameters, except for water temperature in autumn and summer, and dissolved oxygen (DO and % DO) in summer. Significant seasonal differences were found for water temperature in both rivers, for chemical oxygen demand (COD), total phosphorus and nitrates in the AR and for dissolved oxygen (DO and % DO) and nitrites in the FR (Table 11). In both rivers, all physico-chemical parameters, except nitrites in the AR and water temperature, DO and % DO in the FR, differed significantly among sites (Table 11).

Overall, DO and % DO values were within the limit values for "good" ecological status of Northern Portuguese rivers (i.e., DO ≥ 5 mg O_2/L; % DO: 60 – 120% of saturation) in both studied rivers and in all seasons (Table 11), except for the % DO at all FR sites in summer. In the FR, lower DO and % DO means were observed in the summer compared to autumn and spring (Table 11).

In AR, COD and phosphorus levels were significantly higher in spring compared to the remaining seasons (Table 11). High levels of phosphorus (i.e., P > 0.10 mg/L) were observed at all AR sites in spring, as well as in AR1, AR3 and AR4 in summer. Although nitrate values observed at all AR sites and seasons were considered "good" (i.e., NO_3^- ≤ 25 mg/L), spring values were significantly lower than those observed in autumn and summer (Table 11).

In the FR, high levels of nutrients (i.e., P > 0.10 mg/L; NO_3^- > 25 mg/L; NO_2^- > 0.1 mg/L; NH_4^+ > 1 mg/L) were found in all seasons, especially at the downstream sites (FR4 to FR6). Conductivity, salinity, TDS were also higher at the downstream sites compared to the upstream sites (Table 11). In FR, the amount of nitrites differed significantly between seasons, with higher values occurring in spring compared to autumn and summer (Table 11).

Table 11.

Spatial and seasonal variation of water physico-chemical parameters determined in the Âncora (AR) and Ferreira (FR) rivers. Mean ± standard deviation of water temperature (Temp, °C), pH, dissolved oxygen concentration (DO, mg O_2/L), percent saturation of dissolved oxygen (% DO), chemical oxygen demand (COD, mg/L), conductivity (Cond, µS/cm), salinity (Sal, PSU), total dissolved solids (TDS, mg/L), total suspended solids (TSS, mg/L), total phosphorus (P, mg/L), nitrates (NO_3^-, mg/L), nitrites (NO_2^-, mg/L), ammonium ion (NH_4^+, mg/L), are presented. Different letters identify significant differences ($p < 0.05$) between sites and seasons, as indicated by post-hoc pairwise Wilcoxon rank sum tests.

AR	Sampling site					Season		
	AR1	AR2	AR3	AR4	AR5	Autumn	Spring	Summer
Temp	14.46 ± 2.38^{a}	15.38 ± 2.16^{ab}	15.57 ± 1.99^{bc}	16.39 ± 3.20^{bc}	16.19 ± 2.90^{c}	12.75 ± 0.51^{a}	16.78 ± 1.43^{b}	17.26 ± 0.78^{b}
pH	5.98 ± 1.24^{ab}	5.32 ± 0.16^{b}	5.73 ± 0.22^{a}	5.82 ± 0.16^{a}	5.81 ± 0.16^{a}	5.97 ± 0.85	5.59 ± 0.24	5.63 ± 0.35
DO	9.03 ± 0.49^{a}	9.11 ± 0.81^{a}	7.34 ± 1.76^{b}	7.39 ± 2.75^{ab}	8.03 ± 2.67^{ab}	9.42 ± 1.06	6.46 ± 1.97	8.66 ± 0.81
% DO	90.67 ± 0.68^{abc}	90.97 ± 4.99^{bc}	79.13 ± 11.84^{a}	82.50 ± 11.53^{b}	91.87 ± 7.83^{c}	89.04 ± 9.69	81.34 ± 7.19	90.7 ± 8.55
COD	8.00 ± 13.00^{ab}	4.67 ± 7.23^{ab}	1.00 ± 1.73^{a}	5.00 ± 7.81^{ab}	5.33 ± 5.03^{b}	1.20 ± 2.68^{b}	12.60 ± 7.23^{a}	0.60 ± 0.55^{b}
Cond	21.00 ± 3.61^{a}	35.67 ± 2.31^{b}	39.67 ± 3.21^{c}	44.00 ± 6.93^{c}	45.00 ± 2.65^{d}	37.4 ± 7.37	37.4 ± 10.53	36.4 ± 12.62
Sal	0.01 ± 0.00^{a}	0.02 ± 0.01^{b}	0.02 ± 0.00^{b}	0.02 ± 0.00^{b}	0.02 ± 0.00^{b}	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.01
TDS	10.33 ± 1.53^{a}	17.67 ± 1.53^{b}	19.67 ± 1.53^{c}	22.00 ± 3.46^{d}	22.67 ± 1.15^{e}	18.6 ± 3.85	18.6 ± 5.27	18.2 ± 6.42
TSS	1.61 ± 1.56^{ab}	1.74 ± 0.64^{a}	1.24 ± 1.41^{a}	1.34 ± 1.27^{a}	1.78 ± 1.77^{a}	0.81 ± 0.54	2.61 ± 1.25	1.2 ± 0.92
P	0.19 ± 0.17^{a}	0.11 ± 0.08^{b}	0.12 ± 0.08^{b}	0.26 ± 0.22^{a}	0.10 ± 0.09^{b}	0.05 ± 0.02^{a}	0.29 ± 0.14^{b}	0.12 ± 0.06^{c}
NO_3^-	7.09 ± 6.95^{ac}	8.76 ± 2.72^{ac}	14.13 ± 7.58^{b}	10.09 ± 1.70^{ab}	8.37 ± 2.10^{c}	10.45 ± 7.11^{ab}	6.53 ± 2.85^{a}	12.08 ± 1.74^{b}
NO_2^-	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
NH_4^+	0.59 ± 0.18^{a}	0.47 ± 0.14^{b}	0.42 ± 0.13^{b}	0.32 ± 0.05^{c}	0.27 ± 0.21^{d}	0.43 ± 0.07	0.35 ± 0.20	0.46 ± 0.24

FR	Sampling site						Season		
	FR1	FR2	FR3	FR4	FR5	FR6	Autumn	Spring	Summer
Temp	15.92 ± 4.95	15.48 ± 4.26	15.97 ± 5.32	15.60 ± 4.86	15.33 ± 4.84	15.30 ± 5.32	10.46 ± 0.49^{a}	20.25 ± 0.71^{b}	16.09 ± 0.09^{c}
pH	6.46 ± 0.48^{abc}	6.05 ± 0.17^{ad}	6.50 ± 0.47^{abd}	6.70 ± 0.39^{bc}	6.75 ± 0.26^{c}	6.30 ± 1.89^{d}	6.38 ± 0.23	6.52 ± 0.30	6.66 ± 0.32
DO	7.17 ± 1.76	7.29 ± 1.86	7.32 ± 1.61	6.87 ± 1.12	7.47 ± 1.86	7.09 ± 1.67	8.34 ± 0.41^{a}	7.92 ± 0.51^{a}	5.35 ± 0.18^{b}
% DO	73.87 ± 19.90	74.43 ± 19.34	74.03 ± 17.07	68.47 ± 10.96	73.80 ± 17.64	69.63 ± 13.34	74.92 ± 3.24^{a}	87.22 ± 6.43^{b}	54.98 ± 1.35^{c}
COD	5.67 ± 9.81^{a}	17.00 ± 18.68^{a}	0.00 ± 0.00^{b}	15.00 ± 24.27^{a}	0.00 ± 0.00^{b}	5.00 ± 8.66^{a}	9.67 ± 17.40	9.33 ± 15.10	2.33 ± 5.72
Cond	118.33 ± 12.74^{a}	124.33 ± 21.13^{a}	148.00 ± 26.89^{b}	167.67 ± 14.29^{c}	183.00 ± 10.44^{d}	172.33 ± 8.02^{c}	150.83 ± 31.47	160.33 ± 34.75	145.67 ± 221.77
Sal	0.06 ± 0.01^{a}	0.06 ± 0.01^{a}	0.07 ± 0.01^{b}	0.08 ± 0.01^{c}	0.09 ± 0.01^{d}	0.08 ± 0.00^{e}	0.07 ± 0.01	0.07 ± 0.02	0.07 ± 0.01
TDS	59.00 ± 6.08^{a}	62.00 ± 10.54^{a}	74.33 ± 13.58^{b}	83.67 ± 7.09^{c}	91.33 ± 5.51^{d}	86.00 ± 4.00^{c}	75.17 ± 15.68	80.17 ± 17.51	72.83 ± 10.70
TSS	1.43 ± 1.53^{a}	5.86 ± 4.96^{bc}	3.60 ± 4.32^{ab}	3.89 ± 3.38^{bc}	3.97 ± 3.20^{bc}	6.03 ± 4.01^{c}	1.26 ± 1.09	6.52 ± 3.54	4.62 ± 3.34
P	0.12 ± 0.11^{a}	0.27 ± 0.17^{b}	0.42 ± 0.20^{c}	0.82 ± 0.46^{d}	0.83 ± 0.55^{d}	0.61 ± 0.29^{d}	0.25 ± 0.13	0.56 ± 0.21	0.73 ± 0.57
NO_3^-	22.30 ± 5.95^{ac}	19.34 ± 7.09^{a}	25.20 ± 8.49^{ac}	27.07 ± 2.15^{bc}	31.35 ± 7.57^{b}	27.17 ± 10.39^{c}	25.82 ± 2.99	21.76 ± 4.09	28.65 ± 11.37
NO_2^-	2.56 ± 4.43^{a}	4.34 ± 7.50^{a}	5.69 ± 9.51^{b}	8.93 ± 13.93^{c}	13.03 ± 20.21^{c}	10.11 ± 16.07^{c}	0.29 ± 0.22^{a}	21.22 ± 10.68^{b}	0.81 ± 0.96^{a}
NH_4^+	0.30 ± 0.13^{a}	0.64 ± 0.59^{b}	0.80 ± 0.78^{be}	3.14 ± 1.91^{c}	2.29 ± 1.89^{d}	1.12 ± 0.70^{e}	1.45 ± 0.60	0.64 ± 0.69	2.06 ± 2.25

Note: limit values established for the "good" ecological status in Northern Portuguese rivers: DO ≥ 5 mg O_2/L, % DO: 60 – 120 %, P ≤ 0.10 mg/L; NO_3^- ≤ 25 mg/L.

4.4.2. Biological and hydromorphological indices

Scores of the biological ($IPtI_N$) and hydromorphological (HQA and HMS) indices determined for the same sites and seasons as the macroinvertebrates sampling campaigns for biomarkers measurements, were obtained from Chapter 3. Significant differences between rivers ($p < 0.05$) were found for all indices in the different seasons, except for HQA in the summer ($p = 0.0541$), with the AR reaching higher $IPtI_N$ and HQA scores and lower HMS scores than the FR (Table 12). In both rivers significant differences between sites were found for the biological and hydromorphological indices determined, but not among seasons.

Table 12.

Spatial and seasonal variation of biological (North Invertebrate Portuguese Index, $IPtI_N$) and hydromorphological (Habitat Quality Assessment, HQA; Habitat Modification Score, HMS) indices determined in the sampling sites of the Âncora (AR) and Ferreira (FR) rivers. Data are presented as mean ± standard deviation and different letters identify significant differences ($p < 0.05$) between sites and seasons, as indicated by post-hoc pairwise Wilcoxon rank sum tests.

AR	Sampling site						Season		
	AR1	AR2	AR3	AR4	AR5		Autumn	Spring	Summer
$IPtI_N$	0.82 ± 0.08^{a}	0.76 ± 0.02^{ab}	0.71 ± 0.14^{bc}	0.73 ±0.08^{bc}	0.71 ± 0.08^{c}		0.67 ± 0.07	0.78 ± 0.08	0.78 ± 0.07
HQA	67.00 ± 2.31^{a}	71.00 ± 1.00^{b}	66.00 ± 0.58^{c}	60.00 ± 0.58^{d}	64.00 ± 1.73^{e}		65.40 ± 4.04	65.40 ± 4.45	65.80 ± 3.77
HMS	420.00 ± 0.00^{a}	885.00 ± 0.00^{b}	0.00 ± 0.00^{c}	715.00 ± 0.00^{d}	845.00 ± 0.00^{e}		573.00 ± 368.52	573.00 ± 368.52	573.00 ± 368.52
FR	Sampling site						Season		
	FR1	FR2	FR3	FR4	FR5	FR6	Autumn	Spring	Summer
$IPtI_N$	0.36 ± 0.15^{abc}	0.37 ± 0.16^{a}	0.38 ± 0.08^{a}	0.33 ± 0.04^{a}	0.30 ± 0.05^{b}	0.25 ± 0.02^{c}	0.31 ± 0.05	0.36 ± 0.11	0.33 ± 0.12
HQA	52.00 ± 3.21^{a}	41.00 ± 1.53^{b}	48.00 ± 2.65^{c}	68.00 ± 2.89^{d}	42.00 ± 5.13^{e}	41.00 ± 3.46^{f}	49.00 ± 11.37	47.00 ± 10.18	50.67 ± 10.83
HMS	1045.00 ± 0.00^{a}	1180.00 ± 0.00^{b}	1000.00 ± 0.00^{c}	830.00 ± 0.00^{d}	1285.00 ± 0.00^{e}	1010.00 ± 0.00^{f}	1058.00 ± 157.63	1058.00 ± 157.63	1058.00 ± 157.63

Note: *Ecological quality classes for "small sized streams of North of Portugal"*: *HQA* score ≥ 46, class I (high ecological quality, i.e., high habitat diversity); *HMS* score ≤ 16, class I (high ecological quality, i.e., no artificial modifications in the river channel morphology). *$IPtI_N$*: score ≥ 0.87, class I (high ecological status); score 0.68-0.86, class II (good ecological status); score 0.44-0.67, class III (moderate ecological status); score 0.22-0.43, class IV (poor ecological status); score ≤ 0.21, class V (bad ecological status).

4.4.3. Pesticides, sediment organic carbon and trace elements in the sediment

Calibration curves were performed for standards dissolved in solvent and for matrix standard extracts. Because of the presence of matrix interferences, a matrix-matched calibration curve was more appropriate for the quantification of these pesticides in sediments. Matrix-matched calibration curves for the analysis of pesticides in sediments of both studied rivers were linear over the whole range of concentrations tested for all pesticides, as indicated by the very good values of the coefficient of determination (r2 > 0.99) (Table 13). For all pesticides, limits of detection (LOD) and quantification (LOQ) were in a range from 11.7 to 27.2 ng/g d.w. and 39 to 90.8 ng/g d.w., respectively.

Table 13.

Analytical parameters of the chromatographic method (coefficient of determination; limits of detection, LOD; limits of quantification, LOQ) and mean recoveries ± relative standard deviation of spiked sediment samples (sediments of grain size ≤ 0.25 mm spiked at 50, 100 and 200 ng/g d.w.; sediments of grain size ≤ 2 mm, spiked at 50 and 100 ng/g d.w.), within each pesticide analysed.

				Recovery (%) (n = 2) ± RSD				
				Grain size: ≤ 0.25 mm			Grain size: ≤ 2 mm	
Pesticide	Coefficient of determination	LOD (ng/g d.w.)	LOQ (ng/g d.w.)	50 (ng/g d.w.)	100 (ng/g d.w.)	200 (ng/g d.w.)	50 (ng/g d.w.)	100 (ng/g d.w.)
Dimethoate	0.995	18.85	62.82	83 ± 2	71 ± 10	131 ± 7	99 ± 5	48 ± 9
Diazinon	0.998	11.70	39.01	114 ± 10	109 ± 9	84 ± 8	113 ± 8	85 ± 5
Chlorpyrifos-methyl	0.996	18.36	61.20	94 ± 9	95 ± 5	85 ± 9	89 ± 9	76 ± 9
Parathion-methyl	0.991	27.23	90.78	118 ± 8	110 ± 8	109 ± 10	104 ± 10	79 ± 3
Malathion	0.995	20.55	68.50	115 ± 9	107 ± 7	79 ± 9	100 ± 2	74 ± 7
Chlorpyrifos	0.997	15.36	51.20	104 ± 9	99 ± 2	79 ± 2	92 ± 5	69 ± 6
Chlorfenvinphos	0.997	16.32	54.41	99 ± 10	99 ± 5	118 ± 3	96 ± 7	88 ± 5
Phosmet	0.995	15.34	51.13	–	–	–	–	–

Recovery experiments were performed in duplicate, at three concentrations (50, 100, 200 ng/g d.w.), for sediment with grain size ≤ 0.25 mm. The results obtained ranged between 71–118% and the values of relative standard deviation (RSD) were 2–10%. The recovery experiments with sediments with larger grain size (≤ 2 mm) showed lower recovery percentages than expected at the level 100 ng/g, mainly for dimethoate and chlorpyrifos. Finally, the ultrasonic bath followed by standard EN 15662 citrate-QuEChERS procedure enabled better recoveries of pesticides with the extraction for sediment samples ≤ 0.25 mm grain size and spiked at 50 ng/g d.w. (recoveries between 83–114%; Table 13). All pesticide analyses were performed with sediment samples of ≤

0.25 mm grain size, and only the presence of chlorpyrifos was detected in both rivers, in AR1 and AR3 from AR and in FR1, FR2 and FR5 from FR (Table 14).

Table 14.

Chlorpyrifos concentration in the sediment of the sampling sites of the Âncora (AR1 and AR3) and Ferreira (FR1, FR2 and FR5) rivers where it was detected. Data are presented as mean ± standard deviation.

Sites	Chlorpyrifos (ng/g of d.w.)
AR1	24.00 ± 0.65
AR3	22.47 ± 0.15
FR1	23.22 ± 1.23
FR2	15.93 ± 0.45
FR5	23.12 ± 0.56

Significant differences between rivers were found for the percentage of Zr (p = 0.0060), Sr (p = 0.0050), Th (p = 0.0084), Pb (p = 0.0352), Zn (p = 0.0263), Ti (p = 0.0446) and Ba (p = 0.0266), with FR reaching higher percentages of these elements (Table 15). Percentages of elements Se, Hg, Au, Co, Sc, Cs, Te, Cd, Ag, Pd, S and Sb were not detected (below the limit of detection at all sites of both rivers).

Table 15.

Percentage of trace elements (Mo, molybdenum; Zr, zirconium; Sr, strontium; U, uranium; Rb, rubidium; Th, thorium; Pb, lead; Zn, zinc; W, tungsten; Cu, cooper; Ni, nickel; Fe, iron; Mn, manganese; Cr, chromium; V, vanadium; Ti, titanium; Ca, calcium; K, potassium; Ba, barium; Sn, tin) present in sediment of the sampling sites of the Âncora (AR1 to AR5) and Ferreira (FR1 to FR6) rivers, in spring. LOD: limit of detection. Percentages of some trace elements determined (S, sulfur; Hg, mercury; Au, gold; Co, cobalt; Sc, scandium; Cs, cesium; Te, tellurium; Cd, cadmium; Ag, silver; Pd, palladium; Sb, antimony, As, arsenic) are not shown because they were below the limit of detection (< LOD) at all sites of both rivers.

Sites	Mo (%)	Zr (%)	Sr (%)	U (%)	Rb (%)	Th (%)	Pb (%)	Zn (%)	W (%)	Cu (%)	Ni (%)	Fe (%)	Mn (%)	Cr (%)	V (%)	Ti (%)	Ca (%)	K (%)	Ba (%)	Sn (%)
AR1	0.003	0.009	0.003	0.001	0.014	<LOD	<LOD	0.002	0.002	0.001	0.020	1.019	0.023	0.159	0.004	0.116	0.100	2.071	0.007	0.006
AR2	0.003	0.007	0.004	0.001	0.020	<LOD	0.002	0.001	0.002	0.001	0.020	0.575	0.013	0.169	0.003	0.065	0.009	3.252	0.003	0.001
AR3	0.003	0.013	0.003	0.001	0.014	0.001	<LOD	0.004	0.004	0.003	0.024	2.148	0.031	0.177	0.009	0.266	0.033	1.925	0.017	0.009
AR4	0.004	0.014	0.004	0.001	0.015	0.001	<LOD	0.005	0.002	0.003	0.028	2.218	0.052	0.216	0.009	0.277	0.033	2.176	0.022	0.006
AR5	0.003	0.008	0.003	0.001	0.014	<LOD	<LOD	0.003	0.002	0.001	0.014	1.044	0.026	0.136	0.005	0.118	0.086	2.008	0.006	0.008
FR1	0.003	0.021	0.009	0.001	0.019	0.002	0.001	0.004	0.002	0.002	0.025	1.089	0.025	0.193	<LOD	0.168	0.114	3.211	0.025	0.002
FR2	0.004	0.023	0.008	0.001	0.017	0.002	0.001	0.004	0.002	0.002	0.026	1.031	0.016	0.210	<LOD	0.202	0.095	2.947	0.020	0.004
FR3	0.003	0.034	0.008	0.002	0.023	0.003	0.001	0.007	0.002	0.003	0.027	1.760	0.031	0.177	0.005	0.302	0.106	3.769	0.022	0.002
FR4	0.003	0.030	0.007	0.002	0.018	0.003	0.007	0.006	0.003	0.003	0.026	2.189	0.031	0.192	0.006	0.300	0.070	2.838	0.020	0.002
FR5	0.003	0.021	0.008	0.001	0.017	0.001	0.006	0.006	0.004	0.004	0.025	3.303	0.064	0.184	0.008	0.291	0.071	3.132	0.029	0.002
FR6	0.004	0.033	0.007	0.001	0.017	0.003	0.001	0.007	0.004	0.003	0.029	3.233	0.041	0.219	0.009	0.322	0.041	2.784	0.022	0.002

4.4.4. Biomarker responses

Significant seasonal and spatial differences were found within each river (identified by stars in Figs. 9, 10, 11) for some of the analysed taxa.

4.4.4.1. Neurotransmission (ChE)

In the AR, the medians of ChE activity were in general higher for *Baetis* spp. (103.6 – 304.3 nmol/min/mg protein, except at AR5 in autumn, with a median of 3.5 nmol/min/mg protein), compared to the other analysed taxa (Figs. 9, 10, 11). Most AR1 taxa presented lower medians of ChE activity in spring and higher medians in summer, compared to the other sampling sites (Fig. 10, 11). In addition, significant seasonal differences in ChE activities ($p < 0.05$) were found for *Boyeria* spp. and *Baetis* spp., with higher activities in summer compared to the spring for *Boyeria* spp., and compared to autumn and spring for *Baetis* spp..

In the FR, *Baetis* spp. and *Caenis* spp. showed higher median values of ChE activity (*Baetis* spp: 36.0 – 185.6 nmol/min/mg protein; *Caenis* spp: 70.8 – 255.3 nmol/min/mg protein) than the remaining taxa (Figs. 9, 10, 11). In this river, *Boyeria* spp. and *Calopteryx* spp. had higher ChE activity in summer compared to the other seasons, and *Baetis* spp. had higher ChE activity in autumn compared to spring and summer ($p < 0.05$).

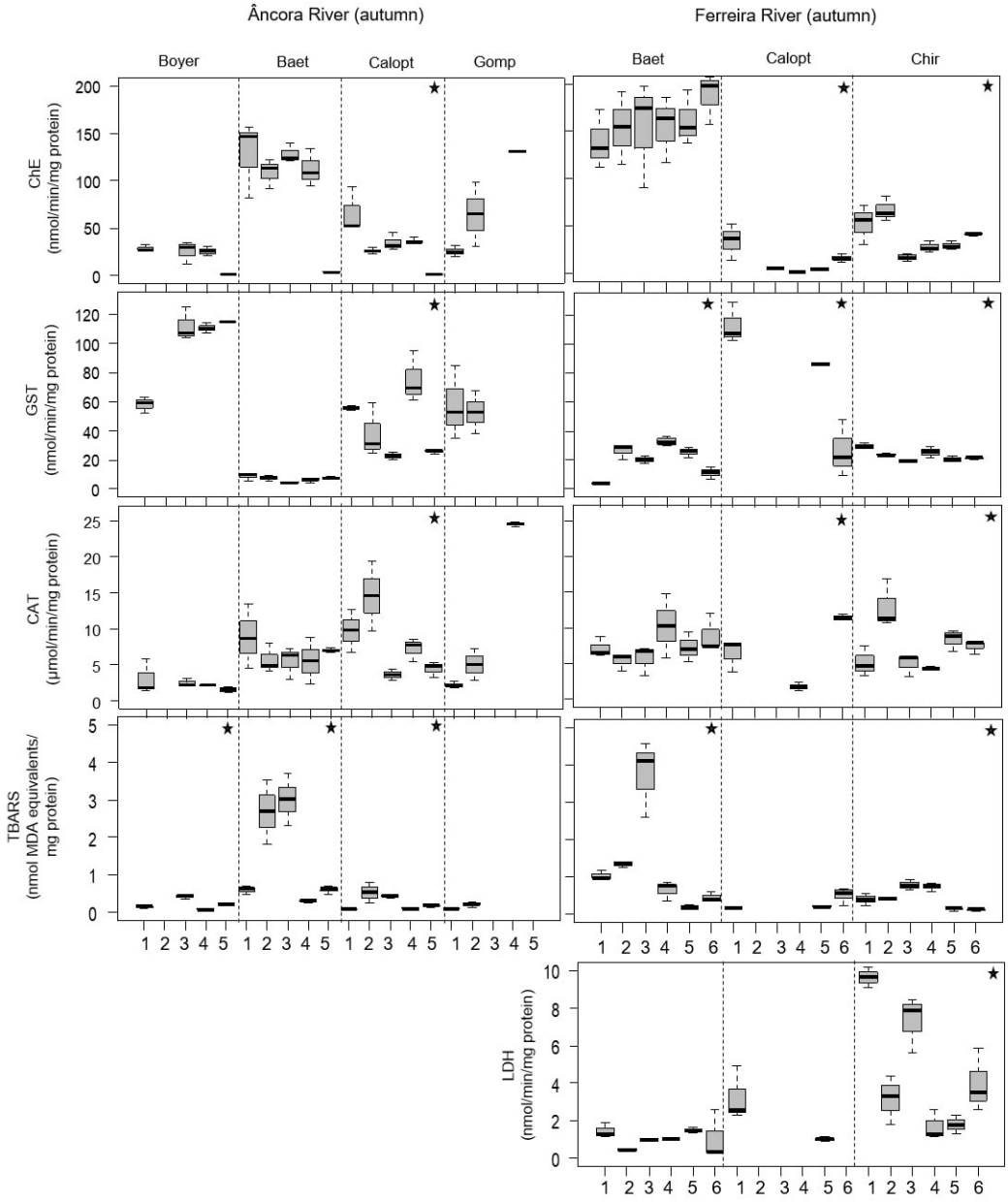

Fig. 9. Box-plots of the enzyme activities (cholinesterases, ChE; glutathione-S-transferases, GST; catalase, CAT; lactate dehydrogenase, LDH) and lipid peroxidation levels (measured as thiobarbituric acid reactive substances, TBARS) in different macroinvertebrate taxa (Boyer: *Boyeria* spp., Baet: *Baetis* spp., Caen: *Caenis* spp., Calopt: *Calopteryx* spp., Chir: Chironomidae, Gomp: *Gomphus* spp.) sampled from the Âncora and the Ferreira river sites (AR1 to AR5; FR1 to FR6) in autumn. The length of each box shows the range within which the central 50% of the values fall, with the box edges at the first and third quartiles. A star identifies taxa for which biomarker activities differed significantly between sites ($p < 0.05$), according to the non-parametric Kruskall-Wallis test.

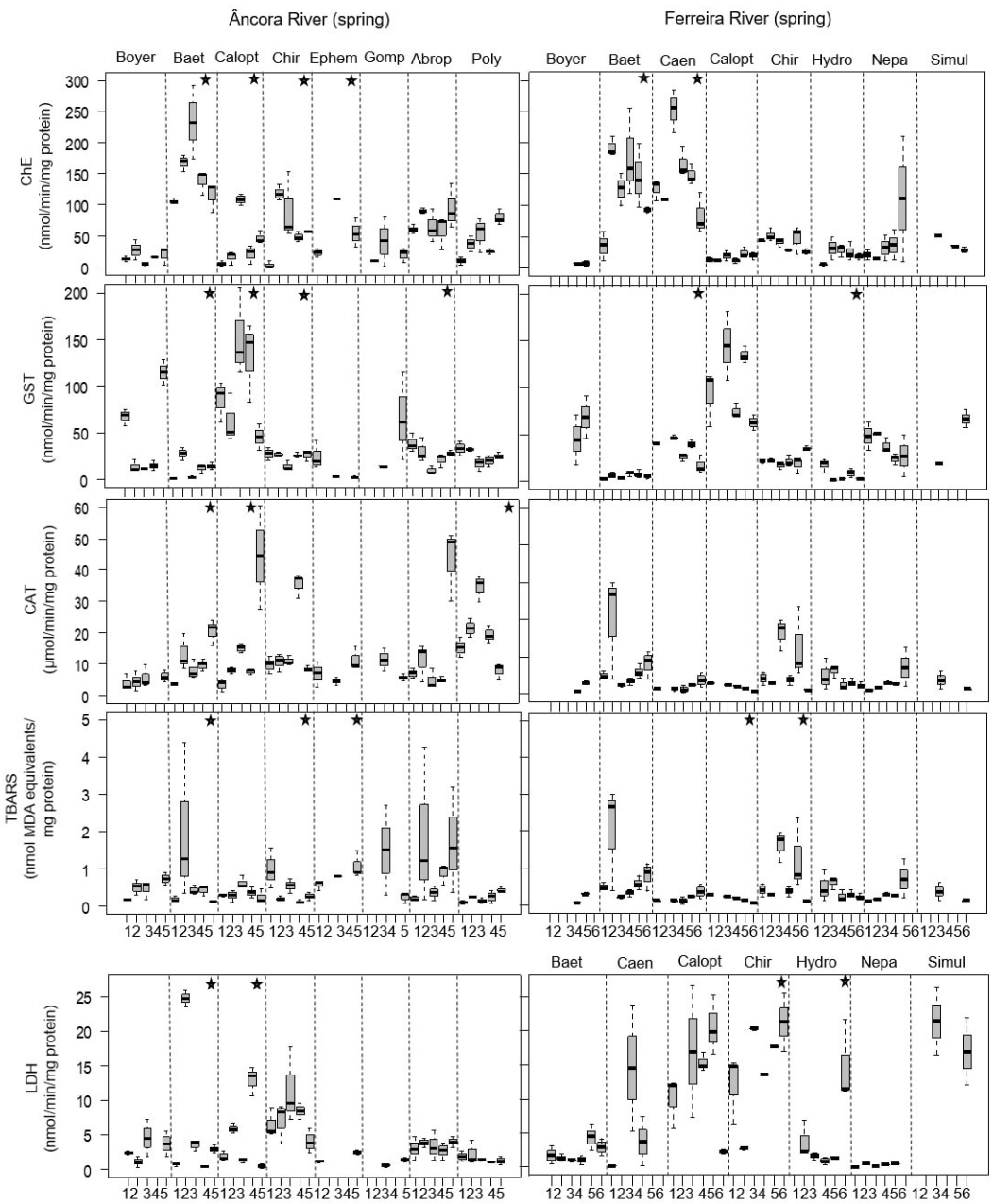

Fig. 10. Box-plots of the enzyme activities (cholinesterases, ChE; glutathione-S-transferases, GST; catalase, CAT; lactate dehydrogenase, LDH) and lipid peroxidation levels (measured as thiobarbituric acid reactive substances, TBARS) in different macroinvertebrate taxa (Boyer: *Boyeria* spp., Baet: *Baetis* spp., Caen: *Caenis* spp., Calopt: *Calopteryx* spp., Chir: Chironomidae, Ephem: *Ephemerella* spp., Gomp: *Gomphus* spp., Hydro: *Hydropsyche* spp., Abrop : *Abrophlebia* spp., Nepa, Poly: Polycentropodidae, Simul: Simuliidae) sampled from the Âncora and the Ferreira river sites (AR1 to AR5; FR1 to FR6) in spring. The length of each box shows the range within which the central 50% of the values fall, with the box edges at the first and third quartiles. A star identifies taxa for which biomarker activities differed significantly between sites ($p < 0.05$), according to the non-parametric Kruskall-Wallis test.

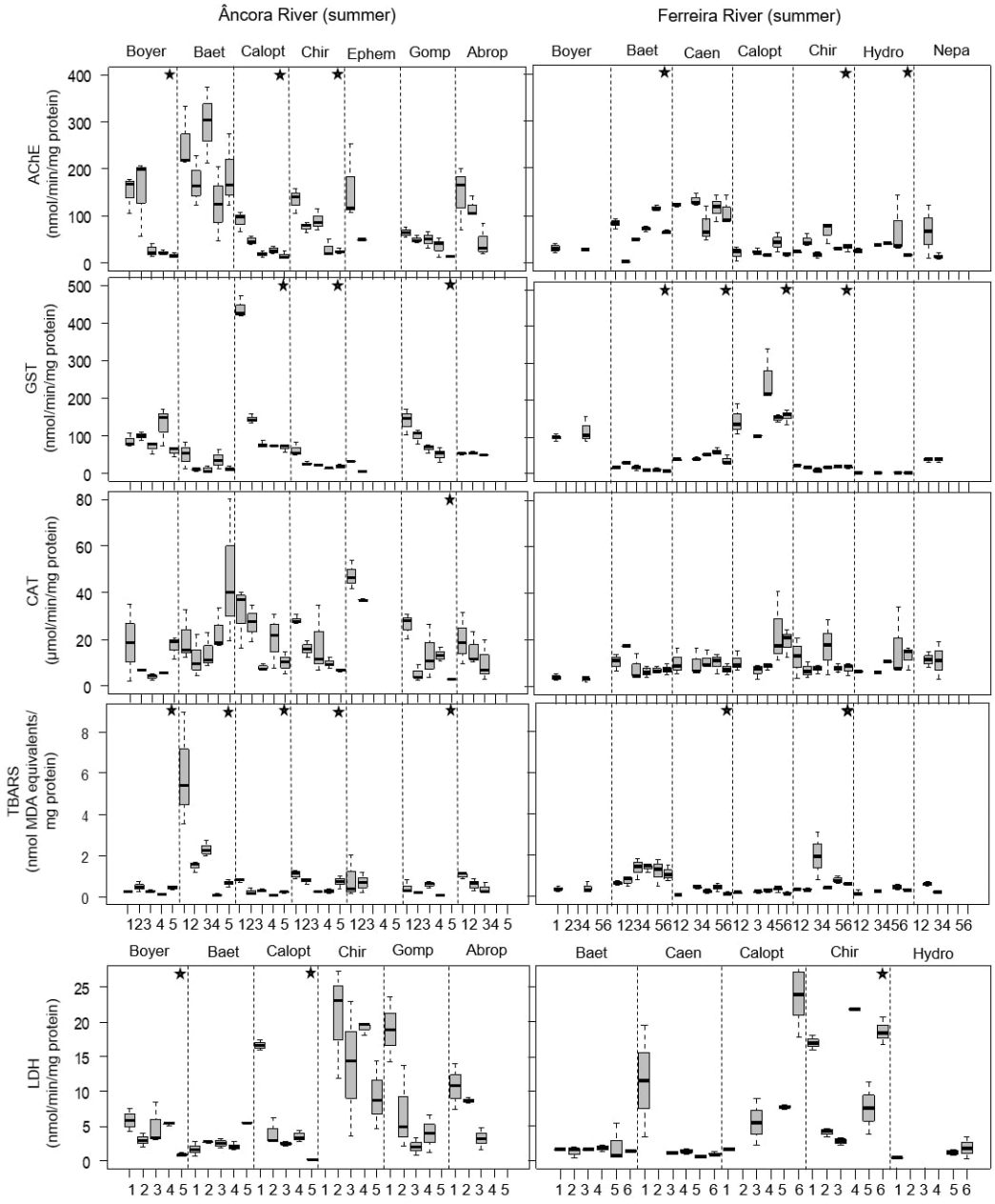

Fig. 11. Box-plots of the enzyme activities (cholinesterases, ChE; glutathione-S-transferases, GST; catalase, CAT; lactate dehydrogenase, LDH) and lipid peroxidation levels (measured as thiobarbituric acid reactive substances, TBARS) in different macroinvertebrate taxa (Boyer: *Boyeria* spp., Baet: *Baetis* spp., Caen: *Caenis* spp., Calopt: *Calopteryx* spp., Chir: Chironomidae, Ephem: *Ephemerella* spp., Gomp: *Gomphus* spp., Hydro: *Hydropsyche* spp., Abrop: *Abrophlebia* spp., Nepa) sampled from the Âncora and the Ferreira river sites (AR1 to AR5; FR1 to FR6) in summer. The length of each box shows the range within which the central 50% of the values fall, with the box edges at the first and third quartiles. A star identifies taxa for which biomarker activities differed significantly between sites ($p < 0.05$), according to the non-parametric Kruskall-Wallis test.

4.4.4.2. Biotransformation enzyme (GST)

In the autumn, the medians of GST activity at AR sites were clearly lower for *Baetis* spp. (4.5 – 6.6 nmol/min/mg protein) compared to the other taxa analysed (26.6 – 107.3 nmol/min/mg protein; Fig. 9). In spring, most taxa presented medians of GST activity below 30 nmol/min/mg protein, but medians above this value were observed, mainly for *Calopteryx* spp. (AR1 to AR5: 46.6 – 135.6 nmol/min/mg protein; Fig. 10). In summer, Odonata (*Calopteryx* spp., *Boyeria* spp., *Gomphus* spp. and *Abrophlebia* spp.) showed medians of GST activity above 40 nmol/min/mg protein at almost all sites, especially at AR1 (Fig. 11). Significant seasonal differences in GST activity ($p < 0.05$) in macroinvertebrates from AR where found for *Boyeria* spp., with higher activities occurring in autumn compared to spring and summer, for *Baetis* spp. and *Calopteryx* spp., with higher values in spring and/or summer compared to autumn, and for *Abrophlebia* spp., with higher values occurring in summer compared to spring.

Higher medians of GST activity were observed in *Calopteryx* spp. in the FR in all seasons and sites (63.1 – 151.8 nmol/min/mg protein, except in FR6 in autumn, with a median of 21.9 nmol/min/mg protein), compared to the other analysed taxa (Figs. 9, 10, 11). In this river, significant differences ($p < 0.05$) in GST activity were found between summer and spring for *Boyeria* spp. and *Caenis* spp., with higher values occurring in summer. There were also significant differences ($p < 0.05$) in GST activity between spring and the remaining seasons in *Baetis* spp., with lower values occurring in spring, and between summer and the remaining seasons in Chironomidae, with lower values occurring in the summe.

4.4.4.3. Antioxidant enzyme (CAT)

The medians of CAT activity in the different taxa analysed in both rivers were mostly below 20 µmol/min/mg protein, although medians above this value were observed for some taxa in spring and summer, in both rivers (Figs. 9, 10, 11). For example, almost all taxa showed a median of CAT activity close to or higher than 20 µmol/min/mg protein at AR1 site in summer (18.5 – 46.7 µmol/min/mg protein, except *Baetis* spp. with a median of 15.7 µmol/min/mg protein; Fig. 11). Significant seasonal differences in the activity of this enzyme ($p < 0.05$) was found in *Boyeria* spp., *Baetis* spp., *Calopteryx* spp. and *Ephemerella* spp. in the AR, and in *Boyeria* spp., *Baetis* spp. and *Caenis* spp. in the FR,

with higher CAT activities occurring in the summer in AR taxa and in the spring in FR taxa.

4.4.4.4. Lipid Peroxidation (measured as TBARS)

In both rivers studied, medians of TBARS levels were generally below 2 nmol/mg protein for all taxa, but higher medians were observed for *Baetis* spp. in both rivers (e.g*.,* at AR1 in the summer and at FR3 in the autumn with a median of TBARS levels of 5.4 and 4.1 nmol/mg protein, respectively; Figs. 9, 10, 11). TBARS levels only varied significantly ($p < 0.05$) among seasons for *Baetis* spp., namely between spring and the remaining seasons in the AR, and between the spring and summer in the FR, with lower levels occurring in the spring in both rivers.

4.4.4.5. Energy metabolism enzyme (LDH)

In most AR taxa*,* medians of LDH activity were, in general, below 10 nmol/min/mg protein in the spring (0.5 – 9.6 nmol/min/mg protein, except in *Baetis* spp. at AR2 and in *Calopteryx* spp. at AR4, with medians of 24.7 and 13.6 nmol/min/mg protein, respectively; Fig. 10). In summer the Chironomidae family showed medians close or higher than 10 nmol/min/mg protein at all sites (8.8 – 26.7 nmol/min/mg protein). In this season, at AR1, most taxa presented activities above 10 nmol/min/mg protein (4 of 6 taxa had medians of LDH activities ranging from 10.8 to 26.7 nmol/min/mg protein) (Figs. 11). In AR, significant seasonal differences ($p < 0.05$) in LDH activity were found for Chironomidae, *Gomphus* spp. and *Abrophlebia* spp., with higher values occurring in summer compared to spring.

For the FR's taxa*,* medians of LDH activity were lower than 10 nmol/min/mg protein in autumn (Fig. 9). In spring and summer (mainly spring), medians above 10 nmol/min/mg protein (11.4 – 31.8 nmol/min/mg protein) were observed for Chironomidae, *Calopteryx* spp. and Simuliidae, at most sampling sites (Figs. 10, 11). In this river, significant seasonal differences ($p < 0.05$) in LDH activity were found for Chironomidae, being this activity lower in autumn compared to the remaining seasons, as well as for *Calopteryx* spp. and *Hydropsyche* spp., with higher values occurring in spring compared to autumn for *Calopteryx* spp., and compared to summer for *Hydropsyche* spp..

Overall, regarding the biomarkers in taxa common to both rivers (Chironomidae, *Baetis* spp., *Calopteryx* spp. and *Boyeria* spp.), significant differences between rivers were found for TBARS levels and LDH activity in *Calopteryx* spp. in spring (TBARS p = 0.0211; LDH p = 0.0472); *Calopteryx* spp. from AR showed higher levels of TBARS those from FR, and *Calopteryx* spp. from FR exhibited higher LDH activities than those from AR. For *Baetis* spp., AChE activities differed significantly between rivers, in both autumn and summer (autumn: p = 0.0062; summer: p = 0.0062). Higher activities were measured in *Baetis* spp. from FR in autumn, and in *Baetis* spp. from AR in the summer. There were also significant differences between rivers in CAT activity for *Baetis* spp. in the summer (p = 0.0090), with *Baetis* spp. from AR showing higher activities than *Baetis* spp. from FR.

4.4.5. Multivariate analysis

The results of the PCA carried out to investigate the overall response patterns of *Calopteryx* spp., *Baetis* spp. and Chironomidae are presented in Figure 12. The first two principle components (PC1 and PC2) were extracted, expressing 50.3% of the total variability observed in the data (Fig. 12).

Biological variables GST and LDH, CAT and $IPtI_N$, and AChE and LPO were found to be highly correlated one to the other, respectively. PC1 established a gradient of response of the taxa examined, opposing *Calopteryx* spp. to *Baetis* spp. (Fig. 12). This component was linked to biomarkers ChE and LPO, and to the macroinvertebrate index ($IPtI_N$). ChE showed a stronger positive correlation with this dimension (r = 0.86, p < 0.001) than LPO (r = 0.67, p < 0.001) and $IPtI_N$ (r = 0.47, p < 0.001). *Calopteryx* spp. appeared to exhibit lower than average levels of AChE and LPO; opposite trends were exhibited by *Baetis* spp. The results also indicated that taxa responses were stable across seasons (Fig. 12). PC2 was linked to GST (r = 0.67, p < 0.001), CAT (r = 0.61 p < 0.001), and to a lesser extent to LDH (r = 0.54, p < 0.001) and $IPtI_N$ (r = 0.44, p < 0.001), all positively correlated with this dimension. *Calopteryx* spp. tended to show higher than average levels of GST, LDH and CAT activities, opposed to the pattern of response of *Baetis* spp.. CAT activity tended to be associated with higher than average levels of the macroinvertebrate index. Overall, the three taxa showed distinct patterns of biological responses, prompting the subsequent analysis of each taxon separately.

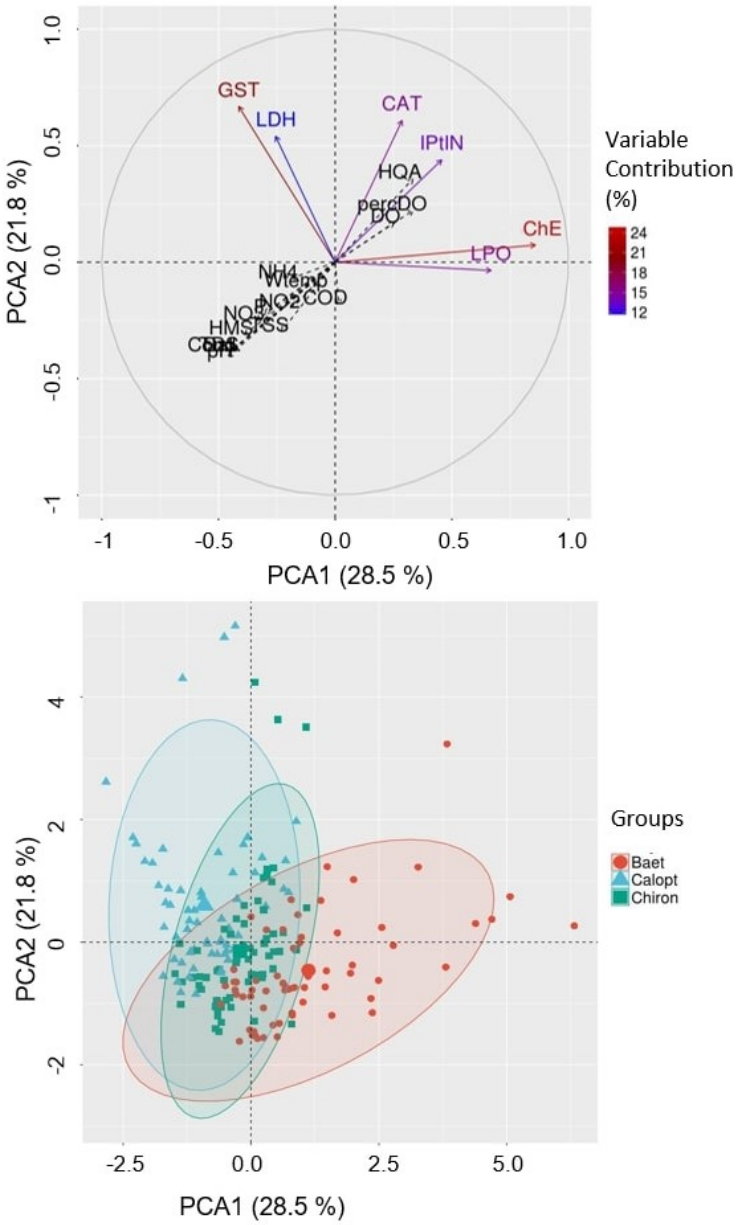

Fig. 12. Results of the PCA carried out with *taxa* and season as qualitative supplementary variables. Qualitative variables were the biomarkers – AChE, GST, CAT, LPO and LDH measured in *Calopteryx* spp., *Baetis* spp. and Chironomidae – and the North Invertebrate Portuguese Index – $IPtI_N$. Water physico-chemical parameters (water temperature, Temp; pH; dissolved oxygen concentration, DO; percent of saturation of dissolved oxygen, % DO; chemical oxygen demand, COD; conductivity, Cond; salinity, Sal; total dissolved solids, TDS; total suspended solids, TSS; total phosphorus, P; nitrates, NO_3^-; nitrites, NO_2^-; ammonium ion, NH_4^+) and hydromorphological indices (Habitat Quality Assessment, HQA; Habitat Modification Score, HMS) were included in the analysis as quantitative supplementary variables. Concentration elipses are shown for each taxa (*Baetis* spp., Baet; *Calopteryx* spp., Calopt; Chironomidae, Chiron).

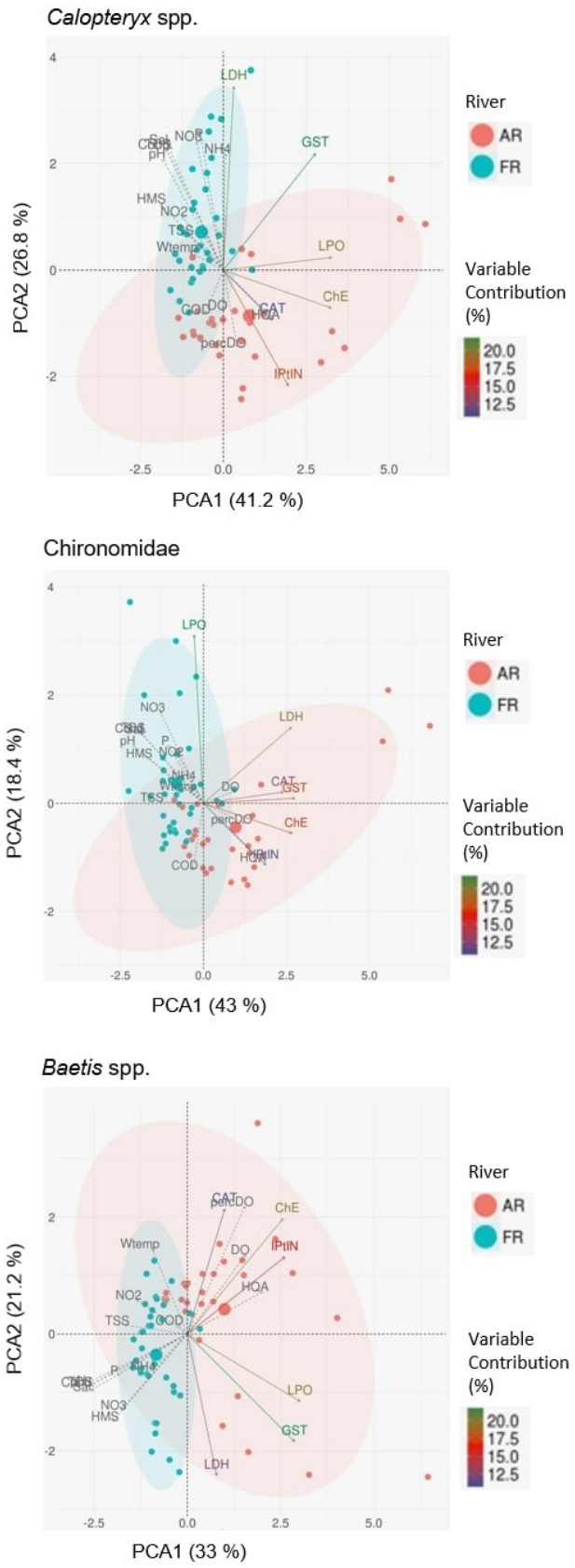

Fig. 13. PCA bioplots showing the relationships among quantitative biological variables, and the spatial gradients established, for each studied taxa. Concentration elipses estimated for each studied river (Âncora River, AR; Ferreira River, FR) are also plotted. Legend as in Figure 12.

All of the three taxa clearly exhibit distinct response patterns for the two rivers studied (Fig. 13). However, PCA based on either *Calopteryx* spp. or Chironomidae explained higher percentages of variability in the datasets. Biological responses were also found to be fairly stable across seasons for all of them. Two significant dimensions were extracted for *Baetis* spp. and Chironomidae, representing 54.2% and 61.4% of the overall variability in the dataset, respectively. Three significant dimensions were extracted for *Calopteryx* spp., expressing 85.2% of the total variability in the dataset. Overall, for each taxon, PC1 and PC2 summarised a representative amount of the total total variance of the dataset (Fig. 13). Hence, interpretation of results was based on these two components. For *Calopteryx* spp., PC1 established a spatial gradient between the rivers, with LPO, AChE and GST showing strong correlations with this dimension (Fig. 13, *top*). Although, less strongly, $IPtI_N$ and CAT were also significantly correlated with PC1. *Calopteryx* spp. from FR were under environmental stress, i.e. exposed to higher than average levels of nitrates, nitrites, ammonia, phosphates and pH. In FR, this taxon exhibited inhibition of ChE and CAT activity, unlike in AR. As previously observed, $IPtI_N$ values were also higher in AR indicating better environmental quality of this water course, compared to FR. Conversely, high LPO levels also tended to occur in AR, suggesting that *Calopteryx* spp. were under chemical stress. Analysis of the cloud of individuals in the dataset revealed that AR macroinvertebrates showing higher LPO levels were collected at the AR1 and AR2 sampling sites. PC2 further distinguished the two rivers. Biological variables correlated with this dimension were LDH, GST and $IPtI_N$. *Calopteryx* spp. from FR exhibited high LDH and GST activity, particularly those from FR5 and FR6 sites, which presented low environmental quality as indicated by low scores of the $IPtI_N$ index (Fig. 13, *top*). In contrast, for Chironomidae, LDH, ChE, and CAT (correlated mainly to PC1) and LPO (correlated to PC2) were the biomarkers producing discrimination of the two rivers (Fig. 13, *middle*). Biomarkers associated to PC1 were also associated to higher environmental quality and ecological status in AR. In contrast, FR tended to show inhibition of these biomarkers and higher LPO levels, particularly in some of the sites.

4.5. Discussion

Nowadays, it is widely accepted that new ecological perspectives for the Water Framework Directive (WFD) require holistic and multidisciplinary approaches integrating multiple lines of evidence. In this study, we aimed to understand if a battery of widely

recognized biochemical biomarkers, evaluated in different macroinvertebrate groups tolerant and sensitive to pollution, could complement the ecological approaches currently applied in assessing and monitoring the aquatic environment. All the biochemical parameters used in this study could be altered by xenobiotic exposure, and were already successfully used as environmental biomarkers in field studies using benthic macroinvertebrates (e.g. Barata et al. 2005; Berra et al. 2004; Kaya et al. 2014; Minutoli et al. 2013; Olsen et al. 2001). Most of these studies, employed one single species for biomarker determination. However, since different taxonomic groups have different sensitivities to environmental stressors, the selection of the most adequate bioindicator species/taxon is not straightforward. Hence, this study adopted a multi-taxon and a multi-biomarker evaluation to identify the most informative taxa, together with a consolidated evaluation of the macroinvertebrate community index recommended by the WFD and evaluation of water physico-chemical quality.

4.5.1. Environmental Parameters

Concerning the water physico-chemical parameters, in the AR, levels of phosphorus above the maximum limit value considered “good” for Northern Portuguese rivers (P > 0.10 mg/L; INAG 2009) were observed mainly in the spring, when increased application of fertilizers in agricultural lands close to the river banks occurred. In the FR, the high levels of nutrients (i.e. P > 0.10 mg/L; NO_3^- > 25 mg/L; NO_2^- > 0.1 mg/L; NH_4^+ > 1 mg/L; INAG 2009) found in all seasons and sites, particularly at the downstream sites (FR4 to FR6) in spring and summer, seemed to be related not only to agricultural practices, but also to discharges of urban areas and wastewater treatment plants.

Organophosphorus pesticides (OPs) are acetylcholinesterase inhibitors and have been largely used because of their efficiency as an insecticide (Girard 2013). Regarding OPs analysis in the sediment of the rivers studied, sediment grain size proved to be an important parameter for the extraction and determination of the pesticides studied (higher recoveries were obtained grain size ≤ 0.25 mm compared to the grain size ≤ 2 mm), and only chlorpyrifos was present in 40% and 50% of the AR and FR sampling sites, respectively. Chlorpyrifos is considered a pseudo-persistent organic pollutant due to its extensive usage and continuous introduction into the environment (Barceló and Hennion 1997; Bonansea et al. 2013; Li et al. 2014). This pesticide has generalized urban and agricultural uses, being applied over all types of crops and even as soil powder for insect control, and it has been used as a substitute for other organophosphate pesticides (such

as chlorfenvinphos, diazinon, parathion-methyl) banned by the EU (Regulation EC No 2009/1107) (Terrado et al. 2009).

Sediments which are habitat and shelter for benthic macroinvertebrates, also represent the major repository for persistent chemicals (organic and inorganic) that reach surface waters (Bettinetti et al. 2012). The Directive 2013/39/EU (EU 2013), which regards priority substances (PS) in the field of water policy, establishes 45 priority substances and sets Environmental Quality Standards (EQS) for these pollutants, including chlorpyrifos (with a maximum allowable concentration of 0.1 μg/L). This Directive has also biota EQS for some PS (biota EQS are related mainly to fish for most PS; but for a few PS, biota EQS refer to crustaceans and molluscs or to all three groups). Biota EQS were specifically set for PS with enhanced hydrophobic properties, and for these PS, biota becomes the "default" monitoring matrix (EU 2013). The promotion of the "default" use of a suitable biota matrix as an alternative to water provides flexibility and cost-effectiveness for the laboratories, and can offset the analytical challenges of nearly non-detectable levels of several PS in water, as a result of their hydrophobic nature (Dosis et al. 2017). However, EQS for pesticides in sediments (as well as soil or sludge) are not included in any directive (Kvičalová et al. 2012) even though the need to do it is recognized by the EU (EU 2013).

The potential ecological risk associated with OP pesticides should not be neglected, but rather monitored as pesticides may cause damage to organisms from the benthic environment (Montuori et al. 2016). There are only a few studies in Europe that determine the occurrence of currently used pesticides in environmental compartments other than water (e.g. Ccanccapa et al. 2016; Cembranel et al. 2017; Hunt et al. 2016; Masiá et al. 2015, 2013; Montuori et al. 2015). Regarding the results of those studies, chlorpyrifos is a frequently detected pesticide in the sediments of European rivers, at lower (e.g. Cembranel et al. 2017; Masiá et al. 2013; Montuori et al. 2015) and higher (e.g. Ccanccapa et al., 2016; Masiá et al. 2015) concentrations than those observed in the AR and FR (mean value of the samples that presented chlorpyrifos: 23.2 ng/g in AR and 20.8 ng/g d.w. in FR). For example, Masiá et al. (2015) reported that chlorpyrifos was present in 7% and 93% of the samples collected from Llobregat River (Spain) in September/October 2010 and October/November 2011, respectively, with mean concentrations of 0.39 and 26.13 ng/g d.w., respectively (concentrations up to 131 ng/g d.w.; Masiá et al. 2015). Ccanccapa et al. (2016) also found this pesticide in all sediment samples collected from Júcar River (Spain) in September/October2010 and October/November 2011, with median concentrations of 2 and 3.15 ng/g d.w., respectively, and in 22% and 11% of the sediment samples collected from the Tugar River in September/October 2012 and October/November 2013, with median

concentrations of 63.75 ng/g d.w and below 0.01 ng/g d.w., respectively (Ccanccapa et al. 2016).

The analysis of pesticides in the sediments of both rivers was carried out based on a single campaign in spring. However, long-term data is essential to assess global changes in fluvial systems, since the transport of pesticides to other environmental compartments such as sediments is affected by aspects that change over time, such as water body characteristics (e.g. depth and flow), proximity of crop fields to surface waters, and climatic conditions (e.g. temperature, wind and precipitation) (Ccanccapa et al. 2016).

Several of the elements that were present in sediments reflect the industrial and other activities in surrounding areas. Some fertilizers and pesticides used in agriculture near the river banks of both rivers studied contain elements such as K, Fe, S, Ca, Mg, and Cu. Higher percentages of some heavy metals, namely Zr, Th, Pb, Zn, Ti and Ba, were found in the FR compared to the AR, as a result of higher anthropogenic pressures in the FR (e.g. higher industrial activity). Sediments of both rivers were mainly composed by coarser particles (granule and pebble, very coarse and coarse sand: > 80% in AR and > 60% in the FR; Chapter 3) to which trace metals have low affinity, thus contributing to the low retention of trace elements in sediments (Eggleton and Thomas 2004). Although the percentage of heavy metals in the sediment of both rivers was low or not detected, a quantitative analysis of trace elements (e.g. using the analytical technique ICP-MS) is necessary since it is much more sentitive than the quanlitative analysis used is this study, and heavy metals (most of the trace elements analysed) are highly persistent and can be toxic to life even in trace amounts.

Regarding the hydromorphological characterization of the sampling sites, high physical habitat heterogeneity was found at all AR sites (except AR3 in summer 2014) and at FR1, FR3 and FR4 from FR. However, channel morphology was artificially modified, especially at AR2, AR4 and AR5, and at all FR sites.

4.5.2. Biological parameters

According to the $IPtI_N$ index based on the macroinvertebrate community, AR sites presented “good” or “high” ecological status in almost all studied seasons (AR3 and AR5 had “moderate” ecological status in autumn), while FR sites presented ecological status below “good” in all seasons (from “moderate” to “bad”; Chapter 3). Although FR sites showed lower ecological status than AR sites, not all taxa common to both rivers showed significant differences between rivers regarding biomarkers responses. *Baetis* spp. and

Calopteryx spp. showed significant differences between rivers regarding some biomarkers activities/levels, although not in all seasons, while Chironomidae and *Boyeria* spp. showed similar biomarker responses in both rivers, for all seasons.

Overall, the here evaluated biomarker responses observed in macroinvertebrate taxa showed generally low levels of variation, though they differed between rivers. They also were of lower magnitude compared to those observed in other studies in which the same biomarkers were measured in macroinvertebrates sampled from polluted Mediterranean rivers (Barata et al. 2005; Damásio et al. 2011; Prat et al. 2013; Puértolas et al. 2010). ChE and CAT activities found in this study were within the ranges reported by Berra et al. (2004), who performed the first attempt to evaluate the basal-level activities of ChE, CAT and GST in different macroinvertebrate families collected from two Italian rivers. However, the GST activities observed in this study were in general of lower magnitude than those reported by Berra et al. (2004). It is well known, though, that species and/or populations of distinct geographic regions, genetic make-up and/or previous history of exposure to environmental contamination may exhibit differences in biochemical and physiological parameters (Boets et al. 2012, Jin et al. 2012). This further highlights the importance of characterising local baseline responses and the need for site-specific evaluations.

Results of this study showed that specific activities of biomarkers varied from taxon to taxon, as previously observed in other studies (Berra et al. 2004; Bonzini et al. 2008), as species' response to pollutants differs depending on their trophic level, habitat type, feeding habits, biotransformation capabilities, and abiotic factors (Barreira et al. 2007). For example, organisms of two genera of the Ephemeroptera order, considered to be in the mid-range for tolerance to most of the environmental stressors (Harrington and Born 2000; Menetrey et al. 2008), namely, *Baetis* spp. (analysed for both rivers) and *Caenis* spp. (analysed only for the FR), showed higher ChE activities compared to the other taxa analysed. The differential sensitivity of ChE found for various taxa suggests they may respond with different intensities when in contact with substances able to interfere with the enzyme activity, probably due to its different inhibition constant (Berra et al. 2004). Another example is that organisms of the Odonata order analysed in both rivers (*Calopteryx* spp., *Boyeria* spp., *Gomphus* spp. and *Abrophlebia* spp.), which are considered sensitive to organic pollution (Alba-Tercedor and Sánchez-Ortega 1988), displayed higher GST activities (especially *Calopteryx* spp. in the FR) in all seasons compared to the other taxa analysed. Higher GST activities in Odonata compared to other benthic macroinvertebrate taxonomic groups (e.g. Ephemeroptera, Diptera, Plecoptera and Trichoptera) were also observed by Berra et al. (2004), and this seems

to indicate that Odonata have greater biotransformation capabilities, compared to other taxa.

In both rivers studied, significant spatial differences in biomarker responses within the same season were observed for some taxa. Nevertheless, a single analysis of the responses observed was not able to differentiate sites with different ecological status, as happened in other studies (e.g. Damásio et al. 2011). There were, however, situations in which biomarkers detected biological sub-lethal effects of chemical or other stress exposure (i.e. effects that are not detected at a structural community level) rather than those associated with high nutrient concentrations and habitat degradation, thus complementing the information given by the ecological quality monitoring procedures currently used in the scope of the WFD. In autumn AR2 and AR5 sites had “moderate” ecological status, according to the $IPtI_N$ index, while the remaining sites had “good ecological status” (Chapter 3). At AR5 in autumn, all macroinvertebrate taxa analysed (including *Baetis* spp.) showed decreased ChE activities compared to the other AR sites, which seemed to be associated with pesticide inputs resulting from ground leaching by the autumnal rains (agricultural land near the river banks) or to interactions of contaminants and environmental factors. Apparently, this contamination did not persist in spring and summer, indicating that pollution sources responsible for the observed biochemical changes were not acting continuously. None of the studied organophosphorus pesticides was found at AR5 in spring. In this season, only AR4 did not show “good” ecological status (Chapter 3) but higher inhibition of ChE was observed at AR1 (compared to the other AR sites), where the highest concentration of chlorpyrifos was also detected (24 ng/g). The pattern of ChE inhibition found in most taxa was not observed at AR3 or in FR sites, where chlorpyrifos was detected at apparently lower concentrations. In fact, the occurrence of a particular contaminant in the environment does not necessarily mean that it is bioavailable, nor can any conclusion be drawn with regard to any resultant harmful effects, or indeed any measurable effects, on biological systems (Lam 2009). Therefore, the biomarkers approach is useful to complement chemical analysis, providing early-warning information about the exposure and/or effects of contaminants on organisms and the possible need for more detailed investigations to be carried out. The AR1 site in summer was the only AR site/season with “high” ecological status (other sites had "good” ecological status). Here, higher median values of activities of enzymes responsible for the normal neural function (ChE) were found, compared to the remaining sites and seasons; although AR1 also showed higher levels than other sites of biotransformation (GST), antioxidant (CAT) and metabolic (LDH) enzyme activities in most taxa analysed. In this regard, it is noteworthy that several forest fires occurred in a nearby area of AR1 in the summer. Deposition of atmospheric

particulate matter resulting from organic combustion may have triggered the activity of those enzymes to cope with the exposure. Although TBARS levels at all sites were of low magnitude, compared to those observed in macroinvertebrates from polluted rivers and streams (Barata et al. 2005; Damásio et al. 2011; Prat et al. 2013; Puértolas et al. 2010), the highest TBARS levels recorded in this study occurred in *Baetis* spp. at AR1 in summer.

4.5.3. Multivariate Analysis

Results suggest that the use of a battery of well-established biomarkers, measured in different macroinvertebrate taxa with different sensitivities to environmental stressors, provides a more integrative and complementary view of ecosystem health than the use of a single taxon, by encompassing diverse forms of biological integration of the environment, multiple exposure routes and different taxa sensitivities. However, for a continuous cost-effective biological monitoring of rivers, the choice of adequate taxa and sampling seasons is relevant. *Baetis* spp., Chironomidae and especially *Calopteryx* spp., seemed to be more sensitive in detecting subtle gradients of toxic substances and their effects than the remaining taxa analysed. These organisms were widespread in the studied areas and abundant or bigger-sized, providing sufficient biological material for the measurement of the whole battery of biomarkers, in most or all sampling sites and seasons.

The PCA done to investigate overall response patterns of *Calopteryx* spp., *Baetis* spp. and Chironomidae showed that biomarker responses in all taxa were stable across seasons (summer and spring), and clearly distinguished *Calopteryx* spp. from *Baetis* spp. patterns of biological response to contamination. *Calopteryx* spp. exhibited lower than average levels of ChE and LPO and higher than average levels of GST, LDH and CAT activities, while *Baetis* spp. showed opposite trends.

The PCAs of the biomarkers measured in *Calopteryx* spp. and Chironomidae individually, clearly discriminated FR (with low environmental quality) from AR (higher environmental quality). For example, *Calopteryx* spp. from FR exhibited inhibition of ChE and CAT, as opposed to *Calopteryx* spp. from AR. This taxon also showed higher GST and LDH activities in FR compared to AR, particularly at the downstream sites (FR5 and FR6), which presented the worst $IPtI_N$ scores, as well as higher levels of nutrients.

AChE is an enzyme belonging to the family of cholinesterases (ChEs), responsible for the degradation of acetylcholine, the primary neurotransmitter in the sensory and neuromuscular systems in most animal species. Although AChE is

specifically inhibited by organophosphorus and carbamate insecticides, it can also be affected by non-specific inhibitors (e.g. metals, PAHs, and even emerging pollutants such as pharmaceuticals), causing an over-accumulation of the neurotransmitter acetylcholine, and thus prolonged electrical activity at nerve endings, which may ultimately lead to death (Berra et al. 2006; Damásio et al. 2011; Domingues et al. 2010; Garcia et al. 2000; Payne et al. 1996; Pestana et al. 2009; Santos et al. 2012; Schulz and Liess 2000; Siebel et al. 2010). CAT is one of the most conspicuous and responsive enzymes to reactive oxygen species (ROS) in both vertebrates and invertebrates (Halliwell and Gutteridge 1999). Many chemical pollutants such as pesticides, polychlorinated biphenyls (PCBs) (Orbea et al. 2002; Winston and Di Giulio 1991) and metals (Stohs and Bagghi 1995) cause oxidative stress by enabling the production of ROS (Barata et al. 2005; Sahan et al. 2010). When the balance between the generation of oxyradicals and its elimination by antioxidants is disrupted, oxidative damage will occur (Halliwell and Gutteridge 1999; Lushchak 2011). The GST enzymatic complex acts on detoxification of xenobiotics (e.g. pesticides, PAHs, oils and complex mixtures of pollutants) and is a defence against oxidative damage (Sáenz et al. 2010). LDH is involved in the anaerobic pathway of energy production and its induction is an indication of increased energy demand to readily cope with chemical stress induced by exposure to contaminants (De Coen and Janssen 1997; Jo et al. 2001; Rodrigues et al. 2013; Rodrigues et al. 2015). Thus, its association with previous mentioned enzymes is also reasonable, suggesting that *Calopteryx* spp. from FR, especially at FR5 and FR6, may have been exposed to low concentration of xenobiotic(s) but were able to cope with the exposure by obtaining additional energy for detoxification and antioxidant protection, since no oxidative damage was observed. Lipid peroxidation measured in this study as TBARS levels, is one of the main mechanisms of oxidative stress which leads to tissue damage, deterioration of cellular functions, and changes in the physico-chemical properties of cell membranes (Rikans and Hornbrook 1997). Higher LPO levels were observed in the AR, particularly at the AR1 and AR3 sites, probably as a consequence of forest fires that occurred upstream of AR1 and near AR3, suggesting that the organisms were under chemical stress. The PCAs also revealed little seasonal variation in the biological variables (both the biomarkers and the macroinvertebrate quality index), suggesting that, under the present climate scenario, monitoring in either season may provide sufficient informative data for the weight-of-evidence approach adopted. Future work should hence also focus on further investigating possible seasonal gradients of response occurring in AR and FR during these and the remaining seasons of the year. Successive sampling efforts may allow to obtain enough samples of *Calopteryx* spp. or Chironomidae (these taxa allowed to discriminate responses between rivers and

provided useful information to identify specific sampling sites under higher environmental stress) for this purpose.

As to the taxonomic resolution for biomarkers analysis, though toxic effects on biota are known to be most noticeable at lower levels (Rubal et al. 2009), the identification of benthic macroinvertebrates species is extremely time-consuming and expensive (Marshall et al. 2006). Also, because of morphological immaturity, some individuals cannot be identified to species-level; some specimens may also be cryptic or represent little known groups (Cook et al. 2008; Liu et al. 2003; Pfrender et al. 2010; Weiss et al. 2014). A good example are the immature stages of chironomids. These usually are the most species-diverse and abundant freshwater macroinvertebrates. Nevertheless, their identification to species or even genus level is technically very difficult or impossible using traditional morphology-based methods (Jones 2008). The present results support their determination in higher level taxa as an expedite, rapid and cost-effective approach, useful for integration in monitoring programmes for ecological quality assessment.

Overall, the biological information provided by biomarkers is essential to assess toxic effects and translate the outcomes of the exposure to multiple stressors, including unknown chemical contaminants (Guimarães et al. 2011). Finally, biological monitoring of AR and FR sites should be continued, in order to verify how the state of organisms or ecosystem health is progressing, and to take timely mitigation actions, if necessary. That would help avoiding that biological effects of contaminants may result in irreversible long-term changes, including biodiversity loss due to disappearing of sensitive taxa.

4.7. References

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05

CHAPTER

Influence of anthropogenic disturbances on species richness of benthic macroinvertebrates belonging to the Chironomidae, Baetidae and Calopterygidae families

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Journal-Article

Rodrigues C. Influence of antropogenic disturbances on species richness of benthic macroinvertebrates belonging to the Chironomidae, Baetidae and Calopterygidae families. (manuscript in preparation) – based on Chapter 5.

Influence of anthropogenic disturbances on species of benthic macroinvertebrates belonging to the Chironomidae, Baetidae and Calopterygidae families

5.1. Abstract

In the current era of biodiversity loss, the assessment and management of anthropogenic impacts on freshwater ecosystems becomes a central challenge. In many bioassessment protocols, benthic macroinvertebrates are identified to family- or genus-level, since their identification to species-level is difficult and error-prone. In this sense, DNA-based methods have been promoted as a way to increase taxonomic resolution and, thereby, to improve the performance of bioassessment metrics.

The main aim of this study was to investigate the influence of anthropogenic disturbances on species richness of benthic macroinvertebrates belonging to the Chironomidae, Baetidae and Calopterygidae families. For that purpose, the metabarcoding approach was used to try to identify to species-level macroinvertebrates belonging to the previously mentioned families. These families have different tolerances to environmental disturbances, are widespread in the studied area and are used for the calculation of the North Invertebrate Portuguese Index ($IPtI_N$) recommended by the Water Framework Directive for the assessment of the ecological status of rivers (Chapter 3). Samples were collected from FR sites with different ecological status classification (moderate, poor and bad), previously evaluated using the $IPtI_N$ index (Chapter 3).

Overall, results showed that the differences between sites regarding species richness (when both a 97 and 85% thresholds were employed to cluster sequences), were mainly influenced by the dipterans of the Chironomidae family. Sites with “moderate” and “poor” ecological status presented the lowest and highest species richness, respectively. The site with “poor” ecological status (FR6) stood out from the other sites (FR1 and FR2) for having higher levels of nutrients and poorer habitat diversity in the river channel (Chapter 4), being these important factors for macroinvertebrates with suitable to traits to colonize or persist in that site (especially chironomids).

For metabarcoding macroinvertebrates, standardized thresholds to assign taxa should be established to ensure the reliability of this technique, and to ensure that bioassessment results across studies can be compared. For example, as observed in this study, changes in the similarity threshold used to cluster sequences can lead to variances in the number of different taxa found in a community. This makes it difficult to compare the results of studies utilizing different thresholds. For the success of the applicability of the DNA metabarcoding technique in benthic macroinvertebrates, it is also essential to develop robust reference libraries.

Keywords: DNA-metabarcoding; high-throughput sequencing; macroinvertebrates; bioassessment; species richness; river

5.2. Introduction

Freshwater ecosystems are among Earth's most threatened habitats due to anthropogenic impacts primarily reflected in global and local species loss (Dudgeon 2010; Sala et al. 2000). Benthic macroinvertebrates are one of the various ecological quality elements used under the Water Framework Directive (WFD; 2000/60/EC; EC 2000) for the assessment of rivers' ecological status. These organisms are common and widespread, with high species diversity with varying sensitivity to environmental disturbances (Resh 2007; Rosenberg and Resh 1993), allowing managers to identify impacted sites and decide on restoration measures. Many bioassessment protocols only require macroinvertebrates to be identified to higher taxonomic levels than species (family, genus) due to the lack of information on functional traits, pollution tolerances and niches preferences of many species, and because species-level identification requires extensive taxonomic expertise and it is time-consuming, expensive and laborious (Aylagas et al. 2014; Macher et al. 2016; Wood et al. 2013; Yu et al. 2012). Moreover, morphology-based approaches can introduce biases due to erroneous species classification, especially in the presence of taxa that lack morphological diagnostic characters at the larval and even the adult stages (cryptic species) and damaged specimens (Kochzius et al. 2008).

According to the taxonomic sufficiency (TS) principle (Ellis 1985), the identification of community components should be made up to the level that provides the required information for the purpose of the work (Rubal 2003). Studies have shown that

taxonomic detail has little influence on the interpretation of multivariate benthic macroinvertebrate community data, suggesting that genus, family, or even coarser aggregations provide sufficient resolution for sensitive and accurate bioassessments (e.g. Bailey et al. 2001; Bowman and Bailey 1997; Warwick 1988). However, other authors argue that it is highly beneficial to include precise species-level information to maximize the capability of bioassessment metrics to discriminate effects of stress (e.g. pollution, environmental degradation) (Hawkins 2006; Hilsenhoff 1977; Jones 2008; Pfrender et al. 2010; Stein et al. 2013; Sweeney et al 2011).

Multiple case studies have demonstrated that species identification, using DNA barcoding, may allow assessing biodiversity and freshwater ecosystems degradation in greater detail than traditional morphology-based approaches (Elbrecht and Leese 2015; Jackson et al. 2014; Pilgrim et al. 2011; Stein et al. 2013; Sweeney et al. 2011). Comparing with traditional morphological identification, the DNA barcoding has the advantage of being independent of the users' taxonomic expertise and makes it possible to assign species names to specimens that are challenging (or impossible) to identify in any other way (Elbrecht and Leese 2015; Jackson et al. 2014; Pilgrim et al. 2011; Stein et al. 2013; Sweeney et al. 2011). This method consists in assigning species names to specimens by amplifying and sequencing (using classical Sanger-based Sequencing) a short standardized DNA fragment (the 'DNA barcode') and comparing it against a reference database of sequences already assigned to specific taxa and analysing the similarities between the sequence obtained and the sequence stored in the database (Hebert et al. 2003; Stein et al. 2014). The most commonly used barcode for animals is a 658 bp section of the mitochondrial cytochrome *c* oxidase subunit I gene (mtDNA COI) (Hebert et al. 2003) and the international project Barcode of Life (Ratnasingham and Hebert 2007) aims to generate a complete species identification catalogue for all animal kingdom organisms based on this gene (Gillet et al. 2015). Other barcode genes are proposed for plants, protists, and meiofauna (Creer et al. 2010; Hollingsworth et al. 2009; Medinger et al. 2010; Yao et al. 2010).

Despite the advantages, identifying single specimens using DNA barcoding is rarely included in biomonitoring programs, especially in freshwater environments, mainly because is still quite laborious and expensive, since each specimen has to be processed and sequenced individually (Cameron et al. 2006; Stein et al. 2014). In contrast, a next-generation sequencing technique termed metabarcoding, utilises the same principle as classical barcoding, yet with much higher throughput, allowing the whole samples to be analysed without needing to isolate individual organisms (Creer et al. 2010). Therefore, besides overcoming dependence on taxonomic expertise, this technique allows rapid analyses of several samples and, consequently reduces monitoring costs and allows

large-scale surveys to be performed (Kelly et al. 2014; Yu et al. 2012). This technique has already been tested and proposed in macroinvertebrates for use in freshwater biomonitoring programmes (e.g. Carew et al. 2013; Elbrecht et al. 2017; Elbrecht and Leese 2017; Elbrecht and Steinke 2018; Emilson et al. 2017; Hajibabaei et al. 2011; Hajibabaei et al. 2012).

The main aim of this study was to investigate the influence of anthropogenic disturbances on species richness of benthic macroinvertebrates belonging to the Chironomidae, Baetidae and Calopterygidae families. For that purpose, the metabarcoding approach was used to try to identify to species-level macroinvertebrates belonging to the previously mentioned families which have different tolerances to environmental disturbances, are widespread in the studied area and are used for the calculation of the North Invertebrate Portuguese Index ($IPtI_N$) recommended by the Water Framework Directive for the assessment of the ecological status of rivers (Chapter 3). Samples were collected from FR sites with different ecological status classification (moderate, poor and bad), previously evaluated using the $IPtI_N$ index (Chapter 3).

5.3. Materials and Methods

5.3.1. Sampling strategy and morphological identification

Three sampling sites (FR1, FR2, and FR6) of a Northern Portuguese river, the Ferreira River, were selected for this study (Fig.14).The FR1 and the FR2 sites are located upstream and immediately downstream of a wastewater treatment plant (WWTP; Paços de Ferreira's WWTP), respectively. The FR6 site is located near the confluence of the Ferreira and Sousa rivers.

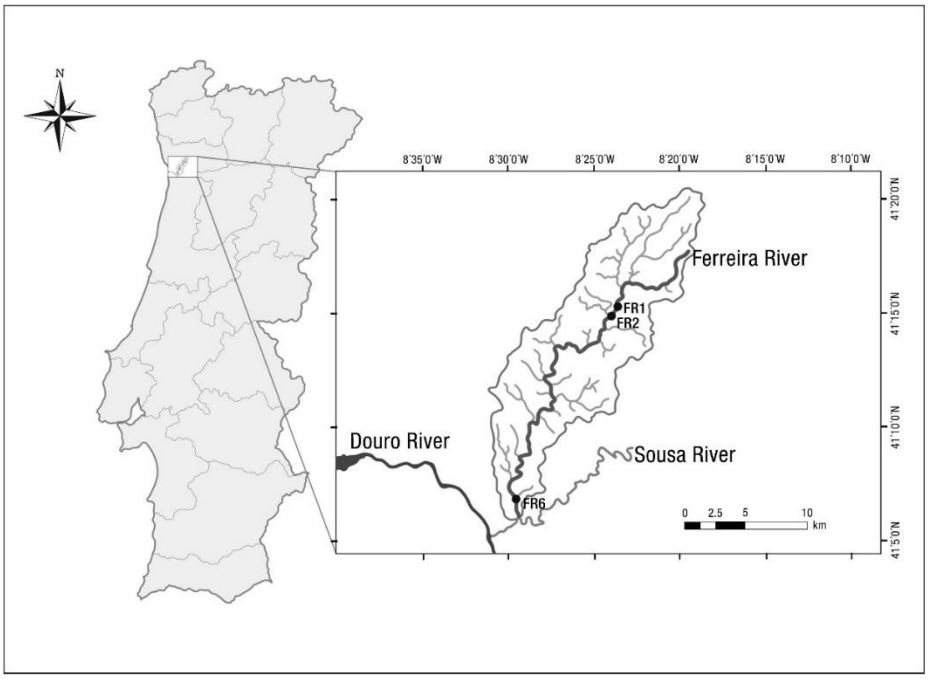

Fig.14. Location of Ferreira River's hydrographic basin in Portugal (mainland) and distribution of the sampling sites within the hydrographic basin.

The selection of the sampling sites was based on their different ecological status previously evaluated (Chapter 3) through the North Invertebrate Portuguese Index ($IPtI_N$; EC 2013; INAG 2009). This index is recommended by the WFD and is suitable for detecting multiple pressure effects in rivers (e.g. organic pollution, acidification, hydrological and morphological alterations and general degradation; INAG 2009).

Sampling campaigns of benthic macroinvertebrate families for species-level identification were performed in the same season (summer of 2014) as the sampling campaigns for the evaluation of the ecological status. Samplings of benthic macroinvertebrates were carried out in triplicate (samples A, B and C) at each site (FR1, FR2 and FR6), following the national guidelines for the WFD implementation (INAG 2008). Insects of the Chironomidae (Diptera), Baetidae (Ephemeroptera) and Calopterygidae (Odonata) families were sorted out from each sample and identified to family-level (required taxonomic level for the calculation of the $IPtI_N$ index) using Tachet's et al. (2002) taxonomic key. These families were found throughout all the sampling sites and exhibit different tolerances to pollution. Chironomidae, Baetidae and Calopterygidae are tolerant, mid-tolerant and sensitive families to organic pollution, respectively, according to the biotic index Iberian Peninsula Biological Monitoring Working Party (IBMWP) included in the multimetric index $IPtI_N$. Benthic macroinvertebrates belonging to these families were also considered suitable for biomarkers analysis for a cost-effective biomonitoring program, providing useful information to identify specific sites

under higher environmental stress (Chapter 4). Organisms were preserved in alcohol (96%) and kept at 8 °C for subsequent molecular analyses.

5.3.2. Molecular analysis

DNA extraction, DNA metabarcoding library preparation and sequencing, quality control and pre-processing of sequencing data, taxonomic assignment and alpha diversity analysis were carried out by All Genetics & Biology, SL. (Coruña, Spain, http://www.allgenetics.eu/).

5.3.2.1. DNA extraction, DNA metabarcoding library preparation and sequencing

Total DNA was extracted from each sample using the DNeasy Power-Soil DNA isolation kit (Qiagen), strictly following the manufacturer's instructions. DNA was ressuspended in a final volume of 100 µL. A DNA isolation blank was included in each extraction round and treated as if it was a regular sample to check for cross-contamination during the DNA extraction procedure.

For library preparation, a fragment of 322 bp within the COI barcode region was amplified, by following a two-step polymerase chain reaction (PCR) approach. PCR1 primers were BF2 (5' GCH CCH GAY ATR GCH TTY CC 3') and BR1 (5' ARY ATD GTR ATD GCH CCD GC 3') (Elbrecht and Leese 2017), to which the Illumina sequencing primer sequences were attached to their 5' ends. The primer set BR2 + BR1 of the COI gene was selected due to its good performance documented in previous studies (Elbrecht and Leese 2017; Elbrecht et al. 2017). PCR2 was carried out with tailed primers that bear the index sequences, which are required for multiplexing different libraries in the same sequencing pool, and annealed to the Illumina sequencing primers. The library was submitted to paired-end sequencing on an Illumina sequencer (MiSeq PE300 run, Illumina).

5.3.2.2. Quality control and pre-processing of sequencing data

Sequencing data were processed using Quantitative Insights Into Microbial Ecology (QIIME) software pipeline (http://qiime.org/). Illumina paired-end raw data contains the demultiplexed FASTQ files, i.e. forward (R1) and reverse (R2) reads with their quality scores sorted by sample. The indices and sequencing primers were deleted during the demultiplexing step.

The quality of the FASTQ files was checked using the FastQC software 0.11.7 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Paired-end assembly of the R1 and R2 reads was performed with FLASH (Fast Length Adjustment of SHort reads; Magoč and Salzberg 2011). The mismatch resolution in the overlapping region (minimum length overlap of 30 base pairs) was accomplished by keeping the base with the highest quality score. The CUTADAPT software 1.3 (Martin 2011) was used to remove sequences that did not contain the PCR primers (allowing up to 2 mismatches) and sequences that ended up being shorter than 300 nucleotides.

The sequences were quality-filtered (minimum Phred quality score of 20) and labelled in QIIME.

5.3.2.3. Taxonomic assignment

An in-house reference database was created with sequences obtained from the Barcode of Life Data System (BOLD) (Ratnasingham and Hebert 2007). All the COI sequences for Baetidae, Calopterygidae, and Chironomidae were retrieved from BOLD and clustered with VSEARCH under an 85% and a 97% similarity thresholds, to cluster filtered reads into OTUs (Operational Taxonomic Units).

The first similarity threshold (85%) was defined taking into account the genetic difference between the COI sequences included in the reference database and the second similarity threshold (97%) was applied following Elbrecht and Leese (2017).

Based on the results of the OTU table, a quality-filtering was carried out, and the OTUs with a number of sequences lower than 0.005% of the total number of sequences were removed (Bokulick et al. 2013), since they can represent ambiguous OTUs generated by PCR and sequencing errors. Only the OTUs that matched any reference sequence in the BOLD database were kept in the OTU table.

5.3.3. Statistical analysis

The differences between sites (FR1, FR2 and FR6 sites) regarding the number of species and genus assigned when a 97% and an 85% thresholds were employed, were evaluated by analysis of variance (one-way ANOVA). In case of rejection of H_0, post-hoc comparisons were performed using Tukey´s test. The significance level was set at 0.05. These analyses were carried out with the Statistical Package for Social Sciences (SPSS) software version 25.0 (IBM Corp. 2017).

5.4. Results and Discussion

Bar charts showing the relative abundance of each OTU in each sample, when sequences were clustered within a 97% and an 85% similarity thresholds, are presented in Figures 15 and 16, respectively.

A total of twelve species were identified through DNA metabarcoding when a 97% similarity threshold was used, namely eleven Chironomidae species belonging to three subfamilies (Chironominae, Orthocladiinae and Tanypodinae) and eight different genus (Chironominae: *Chironomus*, *Parachironomus*, *Paratanytarsus*, *Phaenopsectra*, *Polypedilum* and *Rheotanytarsus*; Orthocladiinae: *Cricotopus*; Tanypodinae: *Ablabesmyia*), as well as one Baetidae species (*Baetis rhodani*; Fig. 15). Baetidae and most Chironomidae species identified (*Baetis rhodani, Chironomus riparius*, *Phaenopsectra flavipes*, *Polypedilum albicorne*, *Polypedilum cultellatum*, *Rheotanytarsus ringei*, *Cricotopus bicinctus*, *Ablabesmyia longistyla*) are already present in Fauna Europaea database, and their distribution includes Portugal mainland (de Jong et al. 2014).

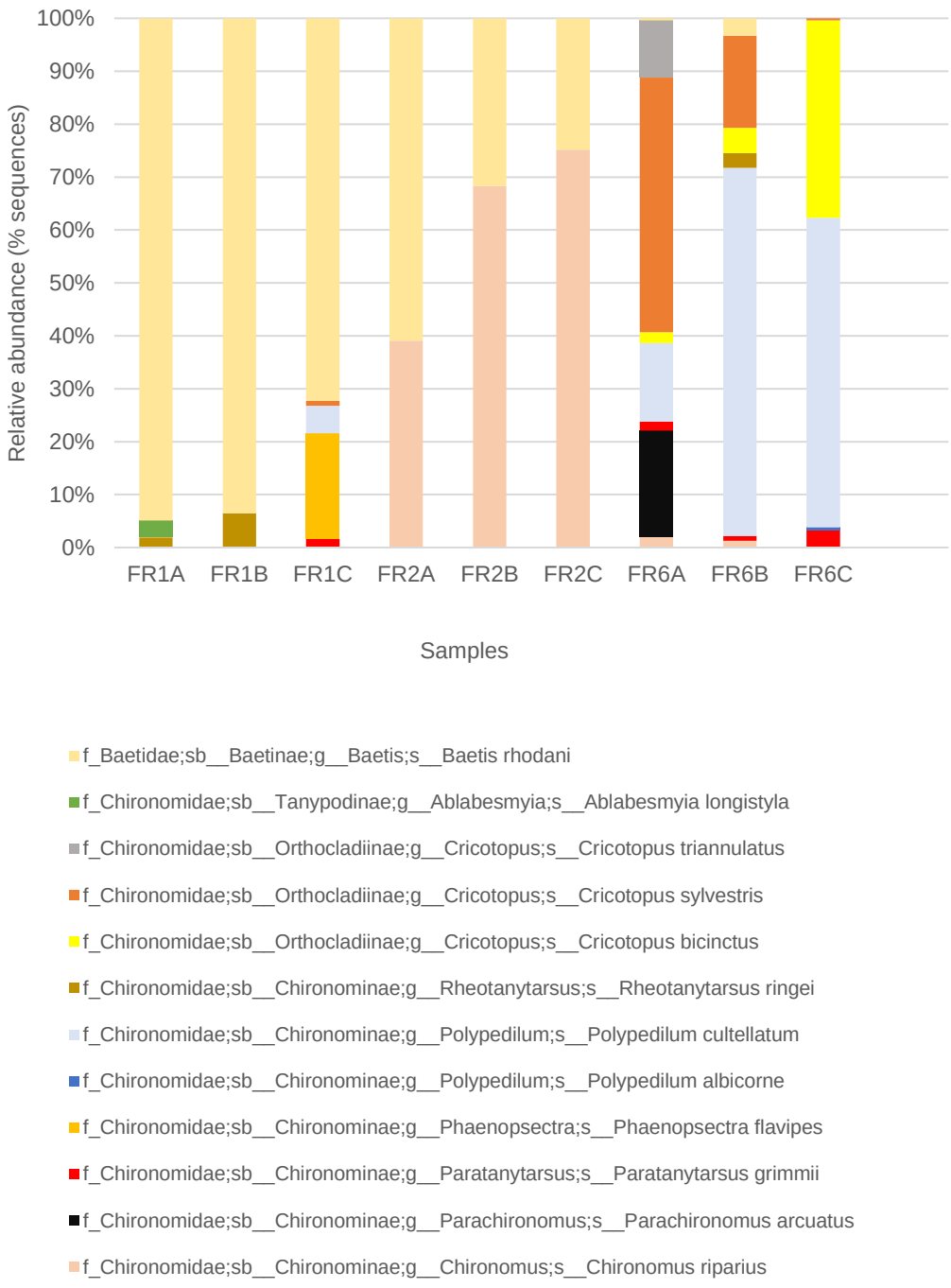

Fig. 15. Benthic macroinvertebrate species assigned (f: family, sb: subfamily; g: genus; s: species) when a 97% similarity threshold was used (f: family; sb: subfamily; g: genus; s: species), as well as and their respective relative abundances (% of sequences in the sample), in each sample (sites: FR1, FR2, FR6; samples A, B and C per site).

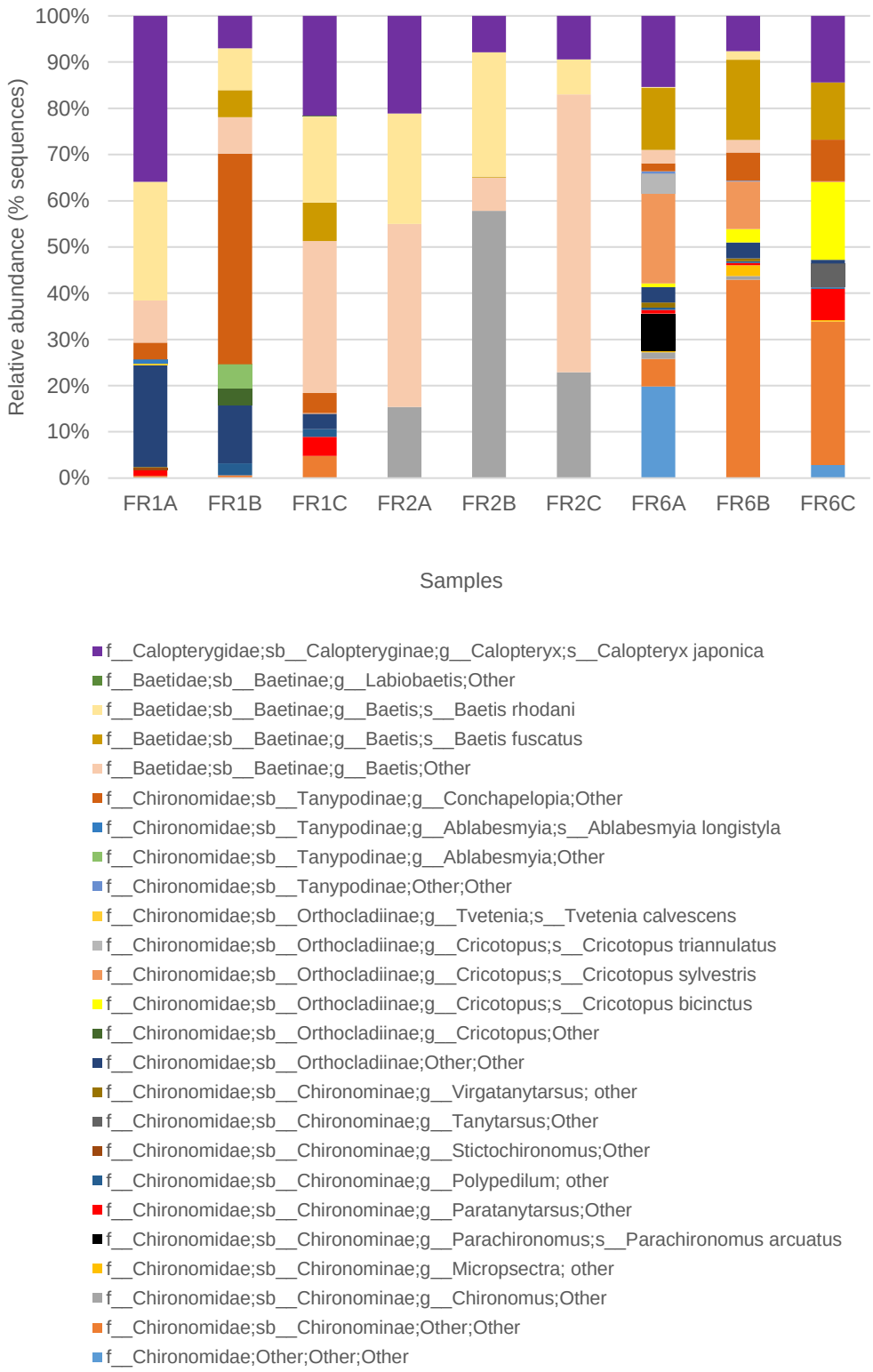

Fig. 16. Benthic macroinvertebrate taxa (f: family; sb: subfamily; g: genus; s: species) assigned when an 85% similarity threshold was used, and their respective relative abundances (% of sequences in the sample), in each sample (sites: FR1, FR2, FR6; samples A, B and C per site).

Some Chironomidae species identified at the FR6 site are present in the Fauna Europaea database but their distribution does not include Portugal mainland (*Paratanytarsus grimmii* present in all FR6 samples; de Jong et al. 2014). Other Chironomidae species identified at the same site are not included in the Fauna Europaea database (*Cricotopus sylvestris* present in all FR6 samples; *Cricotopus triannulatus* and *Parachironomus arcuatus* present in FR6A; de Jong et al. 2014), although European countries are already identified in the map of the collection sites for records in BOLD of those taxa (*Cricotopus sylvestris*: e.g. Norway, Finland, Germany; *Cricotopus triannulatus*: e.g. Germany, Czech Republic, Finland; *Parachironomus arcuatus*: Bulgaria, Finland; Ratnasingham and Hebert 2007), as well as in the map of worldwide occurrence data of those taxa from the Global Biodiversity Information Facility (GBIF; https://www.gbif.org/). A Portuguese island (Funchal, Madeira island) is included in the map of the collection sites for records in BOLD of two identified chironomid species, *Rheotanytarsus ringei* and *Cricotopus bicinctus* (Ratnasingham and Hebert 2007), as well as in the map of worldwide occurrence of those taxa from GBIF (https://www.gbif.org/). Therefore, our findings will contribute to updating the European and global biodiversity databases regarding the insect species that can be found in Portugal mainland.

A total of nine species were identified when an 85% similarity was used to cluster sequences, namely six Chironomidae species belonging to three different subfamilies (Chironominae, Orthocladiinae and Tanypodinae) and 12 different genus (Chironominae: *Virgatanytarsus*, *Tanytarsus*, *Stictochironomus*, *Polypedilum*, *Paratanytarsus*, *Parachironomus, Micropsectra, Chironomus*; Orthocladiinae: *Tvetenia*, *Cricotopus*; Tanypodinae: *Conchapelopia*, *Ablabesmyia*), as well as two Baetidae species (*Baetis rhodani* and *B. fuscatus*; Fig. 16) and one Calopterygidae specie (*Calopteryx japonica*). *Calopteryx japonica* (Calopterygidae) was identified in all samples and *Baetis fuscatus* (Baetidae) was identified in almost all samples (except FR1A, FR2A and FR2C) only when this less stringent similarity threshold of 85% was used (Fig. 14). Portugal mainland was not in the map of the collection sites for records in BOLD of the two previously mentioned species (Ratnasingham and Hebert 2007), as well as in the map of worldwide occurrence data of those taxa from GBIF (https://www.gbif.org/). *B. fuscatus* is already present in Fauna Europaea's database, and its distribution includes Portugal mainland (de Jong et al. 2014). On the other hand, *C. japonica* is not included in this database (de Jong et al. 2014) and has only been recorded in Japan, Russia and South Korea according to GBIF (https://www.gbif.org/). Portugal mainland is in the map of the geographical distribution of three (*Calopteryx haemorrhoidalis, C. virgo* and *C. xanthostoma*), out of four Calopterygidae species already reported in Europe (*C.*

haemorrhoidalis, C. virgo, C. xanthostoma and *C*. *splendens*; de Jong et al. 2014). One of the species that was already identified in Portugal (*C*. *haemorrhoidalis*) does not have a matching reference in the barcode database (Ratnasingham and Hebert 2007), thus not being possible to identify this specie though metabarcoding. Moreover, the number of barcode records for *Calopteryx* species in the BOLD database (i.e. every COI sequence > 500 bp with species-level identification) is much lower (e.g. *C. virgo*: 9 COI sequences; *C. xanthostoma*: 1 COI sequence; *C. japonica*: 1 COI sequence) when compared to the barcode records of the other species identified in this study (from 19 COI sequences, in *Parachironomus arcuatus*, to 2813 COI sequences, in *Cricotopus triannulatus*). This low number of barcode records existent in the BOLD systems for the Calopterygidae family, may not be enough to cover all the intraspecific and interspecific variability within which might lead to mismatches between the OTUs and the reference sequences in the BOLD database, especially when sequences are clustered within an 85% similarity threshold.

Besides *B. fuscatus* and *C. japonica*, some genus belonging to the Chironomidae and Baetidae families were only identified when an 85% similarity threshold was used (*Micropsectra*, *Stictochironomus*, *Tanytarsus*, *Virgatanytarsus*, *Tvetenia* and *Conchapelopia* of the Chironomidae family; *Labiobaetis* of the Baetidae family; Fig. 16).

The results obtained confirmed that the metabarcoding technique improves taxonomic assignment in groups difficult or nearly impossible to distinguish morphologically, such as chironomid larvae (Elbrecht and Leese 2015; Elbrecht et al. 2017). A higher number of Chironomidae species were identified when a 97% similarity threshold was employed. However, some taxa existent in the samples were not assigned to the species-level due to the lack of matching references in the barcode database.

Elbrecht et al. (2017) recommended a similarity threshold of 98% for assignment of benthic macroinvertebrates to species-level, a 95% to genus-level, a 90% to family-level, and an 85% for order-level as a rough proxy. However, the commonly employed thresholds of 97-99% similarity may fail to capture the underlying species composition of an environment, since they are frequently too stringent, producing incorrect estimations of diversity (White et al. 2010). In this study, when a 97% similarity threshold was used to cluster sequences, no Calopterygidae specimens were assigned in any sample and no Baetidae specimens were assigned in the FR6C sample (Fig. 15). Nonetheless, organisms belonging to these families were morphologically identified and their presence was confirmed when a less stringent similarity threshold of 85% was used (Fig. 16).

Regarding the classification of the ecological status of the FR1, FR2 and FR6 sampling sites, they presented “bad”, “moderate” and “poor” ecological status, respectively, according to the $IPtI_N$ index (Chapter 3). This multimetric index requires that

most macroinvertebrates are identified to family-level (class for Oligochaeta) and includes the calculation of measures of richness (number of families of the Ephemeroptera, Plecoptera and Trichoptera orders), diversity (evenness index, i.e. the equitability of Shannon-Wiener's diversity index), composition (abundance of some families), and the IASPT index which is the biotic index Iberian Peninsula Biological Monitoring Working Party (IBMWP) divided by the number of families included in the calculation of this index found in the sample (INAG 2009).

Table 16.

Number of species and genus assigned at each sampling site when a 97% and an 85% similarity thresholds were used to cluster sequences. Data are presented as mean ± standard deviation, and different letters (a and b) identify significant differences ($p < 0.05$) between sampling sites.

	Sampling sites		
	FR1	FR2	FR6
Number of species (97% similarity threshold)	3.3 ± 1.5 (a, b)	2.0 ± 0.0 (b)	6.0 ± 1.7 (a)
Number of genus (97% similarity threshold)	3.3 ± 1.5 (a, b)	2.0 ± 0.0 (b)	6.0 ± 1.7 (a)
Number of species (85% similarity threshold)	3.3 ± 0.6 (a, b)	2.0 ± 0.6 (b)	5.7 ± 1.5 (a)
Number of genus (85% similarity threshold)	5.3 ± 0.6 (a)	3.0 ± 0.0 (b)	7.0 ± 1.0 (a)

Species richness, is an important parameter for resources management considering that distributional ranges of most Earth´s species are declining (Millennium Ecosystem Assessment Board 2005). According to the results obtained, significant differences between FR2 and FR6 sites ($p < 0.05$) were found for the number of species identified when a 97% and an 85% thresholds were used (Table 16). Significant differences between FR2 and FR6 sites (FR2/FR6 $p = 0.024$) and between FR2 and the remaining sites (FR2/FR1 $p = 0.012$; FR2/FR6 $p = 0.001$) were found for the number of genus identified when a 97% and an 85% similarity thresholds were used, respectively (Table 16). Therefore, as observed in this study, changes in the similarity threshold used to cluster sequences can lead to variances in the number of different taxa found in a community.

The differences between sampling sites regarding the number of species and genus (when a 97 and 85% thresholds were employed to cluster sequences; Table 16) were mainly influenced by the dipterans of the Chironomidae family (Figs. 15 and 16). The site with "moderate" ecological status (FR2) was the site with the lower mean of the number of species and genus, when compared to the sites with "bad" (FR1) and especially "poor" (FR6) ecological status (Table 16). The site with "poor" ecological status, was the site with the higher number of species (mainly chironomid species). This site also stood out from the other sites (FR1 and FR2) for having high levels of nutrients

and poorer habitat diversity in the river channel (Chapter 3). There are close to 1300 chironomid species recorded in Europe (Sæther and Spies 2013) and the immature stages of chironomids are commonly the most species-diverse and abundant macroinvertebrates in freshwater ecosystems (Coffman and Ferrington Jr 1996). The ecological amplitude is related to several morphological, physiological and behavioral adaptations found among the members of this family (Coffman and Ferrington Jr 1996). For example, some chironomids have respiratory adaptations (e.g. anal tubules, haemoglobin in Chironominae) to live in anaerobic conditions (Resh and Rosenberg 1984) resulting from eutrophication. The substrate of the river channel at the FR6 site was dominated by sand, thus being mainly colonized by organisms that live buried in the substrate such as chironomids (e.g. Chironominae; Grzybkowska 1992) and oligochaetes (Chapter 3).

The metabarcoding approach is especially relevant for monitoring programs which rely on indices based on the presence/absence of indicator species or ecological groups of species classified according to their sensitivity to stress. Tolerance scores of benthic macroinvertebrates to environmental disturbances are more commonly assigned to family-level although they may vary within lower taxonomic levels (Hilsenhoff 1977) For example, although the Chironomidae family exhibits a wide range of pollution tolerance (Heino and Paasivirta 2008; Roque et al. 2010; Tang et al. 2009), in the IBMWP index (included in the calculation of the $IPtI_N$), all chironomids are considered tolerant to pollution (Chironomidae family has score 2 in a scale of 1 to 10, with the highest scores assigned to species most sensitive to organic pollution; Alba-Tercedor and Sánchez-Ortega 1988).

Regarding biodiversity indices, they combine information about the number of taxa present in a sample with information about the evenness of their counts (e.g. Shannon 1948; Keshler et al. 1978; Washington 1984). At the moment, estimating macroinvertebrate taxa abundance through metabarcoding is challenging due to technical and biological factors. These include PCR biases due to differences in primer specificity, which can cause taxa with a low representation in the original DNA to become more abundant in the final results (Amend et al. 2010; Berry et al. 2011; Deagle et al. 2013; Elbrecht and Leese 2015; Elbrecht et al. 2017; Pinto and Raskin 2012). However, the increase in taxonomic accuracy through DNA-based methods offers an opportunity to investigate potential differences in ecological preferences, to detect the presence of stressors based on indicator species (Macher et al. 2016), and also enables bioassessment metrics to detect subtle changes in the environment (Stein et al. 2014). If chironomids, as well as other taxonomic groups, are identified to species-level instead of family-level, there will be more accurate information on the biodiversity-ecosystem

interactions in freshwater ecosystems, an improvement of the estimates of local diversity of macroinvertebrates, and also more information for conservation biology and environmental assessment.

DNA-based methods have been promoted as a way to increase taxonomic resolution and, thereby, to improve the performance of bioassessment metrics based on taxonomic groups that are currently under-described and under-used. The DNA metabarcoding technique provides fast identification of the entire taxonomic composition of thousands of samples simultaneously (Elbrecht and Steinke 2018; Stein et al. 2014). This is of great relevance considering the increasing water quality monitoring programs in many parts of the world, all requiring a wider number of sampling sites and a larger amount of data (Stein et al. 2014). Although the current economic crisis is leading some countries to monitor their budgets closely (Borja and Elliott 2013), according to some authors, the eventual widespread use of DNA-based identification in biomonitoring studies seems feasible, and costs will probably fall as the technology becomes more mainstream (e.g. Ball et al. 2005; Hebert and Gregory 2005).

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CHAPTER

Main Conclusions

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6.1. Main Conclusions

The main aim of this study was, through an integrative analysis, to approach the possibility of using biomarkers in benthic macroinvertebrates as a complementary tool in the assessment of the ecological status of fluvial ecosystems. This last chapter synthesizes the main conclusions derived from the studies performed in this thesis in order to achieve the main aim.

From the literature review (Chapter 2) it is apparent that, over the last decade, biomarkers have been included in biomonitoring of contamination in rivers and streams using benthic macroinvertebrates. The available literature demonstrates that these tools help to anticipate the detrimental effects of chemical contaminants detected in monitoring programmes. In general, these studies have been based on the use of single and tolerant species, mainly gammarids or caddisfly larvae. Further development of expedite multi-biomarker and multi-taxa approaches may provide new holistic ecosystem-based methods to improve monitoring efficiency.

The most commonly used biomarkers in benthic macroinvertebrates are enzymes involved in neural function and energy production, as well as markers of biotransformation, antioxidant defences and oxidative damage. Although there is an increasing interest in the investigation of emerging contaminants, most studies focused on the so-called legacy contaminants, amongst which metals and pesticides are prominent. Further field research is required to address emerging contaminants and, in particular, to define site-specific baselines taking into account the temporal influence of biotic and abiotic factors. The establishment of robust baseline levels will significantly improve the integration of biomarkers in biomonitoring approaches. It will also favour the estimation of trigger values to address the need for early action in sites with priority mixtures of contaminants which lead to the decline of the ecological status.

Another important aspect is the need for improved understanding on the biomarker-stressor relationships in different macroinvertebrate taxa. This need to be fully tested in new systems, including laboratory tests, determining cause and effect relationships between stressors and biomarkers, and field experiments testing for the strength of these relationships under a variable degree of complexity in natural systems. This will also enlighten knowledge about complex interactions involving exposure levels,

response duration, tissue repair mechanisms and other animal homeostatic processes, and chemical contamination and natural stressors. Finally, standard procedures for biomarker analysis in benthic macroinvertebrate species must also be developed and validated.

Overall, further studies including different biomarkers, environmental stressors, macroinvertebrate taxa and river types will provide the necessary information to establish new, more efficient and cost-effective biomarker and ecological status strategies indicative of future ecological damage.

The ecological status of the Âncora and the Ferreira rivers was primarily assessed through the analysis of benthic macroinvertebrate (North Invertebrate Portuguese Index, $IPtI_N$) and macrophyte (Macrophyte Biological Index for Rivers, IBMR; Riparian Vegetation Index, RVI) communities, as well as physico-chemical and hydromorphological parameters recommended by the WFD (Chapter 3). Macroinvertebrates ($IPtI_N$ index) and macrophytes (IBMR and RVI indices) provided complementary indications for the description of the health status of the studied rivers, providing valuable information for the planning of management actions. The evaluation of the ecological status of rivers using only macrophyte responses to nutrient enrichment (IBMR) provided a partial evaluation of the effects of the stressors affecting the integrity of the river ecosystems. Managers should be warned that this evaluation constitutes an over-simplification and that the evaluation of the ecological status of fluvial systems is best achieved by an integrated multidisciplinary approach, so that a more accurate diagnosis of their ecological status can be obtained.

In terms of season, late spring came out as the most adequate period for monitoring these small Mediterranean rivers, using either the macroinvertebrate-based indices or the macrophyte-based ones. Under fairly stable climate features, this period exhibited greater diversity and abundance of benthic macroinvertebrates, owing to reduced flow impact, as well as more indicator macrophyte species and better survey conditions in terms of water depth and transparency.

Results show that the two studied rivers, which are part of the Natura 2000 Network, need measures to reduce diffuse pollution, as well as restoration of riparian zones, in order to improve their ecological status and preserve their natural habitats and wild fauna and flora. These may encompass measures reducing land use impacts, improving the coverage of the sanitation network and reinforcing/increasing the efficiency of WWTPs treatments. Farmers should also be cautioned against careless and excessive use of fertilizers, and the cleaning of the watercourses, as well as the cutting

of the riparian vegetation, should always be supervised and conditioned in compliance with safeguard measures for the riparian habitats.

A battery of biomarkers of neurotoxicity, biotransformation, antioxidant defences, oxidative stress and energy metabolism was assessed in different benthic macroinvertebrate taxa from both studied rivers (Chapter 4) in order to investigate the potential usefulness of a battery of biomarkers evaluated in different benthic macroinvertebrate taxa to discriminate aquatic ecosystems with different levels of ecological quality and to provide further clues supporting environmental management.

Biomarker responses obtained in both studied rivers indicated that subtle or chronic biological effects may occur in apparently healthy ecosystems, such as the AR (e.g. higher LPO levels in *Calopteryx* spp. and *Baetidae* spp. in AR). The results further point that the use of multiple biomarkers sensitive to water pollution may provide complementary information to diagnose future ecological impairment or to establish reference sites. Results also suggest that a set of well-established biomarkers measured in different macroinvertebrate taxa provides a global complementary view of ecosystem health, since it encompasses diverse forms of biological integration of the environment, multiple exposure routes and different taxa sensitivities. *Calopteryx* spp., Chironomidae and *Baetis* spp. and the spring and summer were the taxa and seasons useful for multivariate analysis, which showed distinct patterns of biological response in the three taxa. The integrated analysis indicated that the most useful taxa to implement a cost-effective application of biomarkers for diagnostic purposes would be *Calopteryx* spp. and Chironomidae. These taxa allowed to discriminate responses among rivers and provided useful information to identify specific sampling sites under higher environmental stress. Looking at these taxa, showing wider distribution, abundant and/or of bigger body size, will help minimize problems such as missing values, high dimensionality, and difficulty in obtaining complete data for some taxa (resulting from their high sensitivity to environmental quality). Each of these taxa presented clear biological response patterns for the two rivers studied, which reflected different ecological status in line with the macroinvertebrate index recommended by the WFD.

A metabarcoding approach was used to identify to species-level benthic macroinvertebrates belonging to the Chironomidae, Baetidae and Calopterygidae families collected from Ferreira River sites with different ecological status classification, in order to investigate the influence of anthropogenic disturbances on species richness on those selected families (Chapter 5). According to the results obtained, a higher

number of macroinvertebrate species were assigned when a 97% was employed to cluster sequences, than when a less stringent similarity threshold of 85% was used. The differences between sampling sites regarding the number of species (when a 97 and an 85% thresholds were used), were mainly influenced by the dipterans of the Chironomidae family. The site with “poor” ecological status presented the highest species richness (especially when compared to the site with “moderate” ecological status). This site also stood out from the other sites (FR1 and FR2) for having higher levels of nutrients and poor habitat diversity in the river channel (Chapter 4), being these important factors for macroinvertebrates with suitable to traits (especially chironomids), to colonize or persist in that site.

For metabarcoding macroinvertebrates, standardized thresholds to assign taxa should be established to ensure the reliability of this technique, and to ensure that bioassessment results across studies can be compared. For example, as observed in this study, changes in the similarity threshold used to cluster sequences can lead to variances in the number of different taxa found in a community. This makes it difficult to compare the results of studies utilizing different thresholds. For the success of the applicability of the DNA metabarcoding technique in benthic macroinvertebrates, it is also essential that both taxonomists and molecular biologists work together using simultaneously morphological and molecular methods in order to develop robust reference libraries, so that the classification of the maximum number of unknown barcodes into species can be performed. It is also necessary to put in place studies on functional traits, niche preferences and tolerance to pollution of the newly identified species, and subsequently to update the existent databases in order to take advantage of all the benefits of species-level identification.

The Marine Strategy Framework Directive (MSFD; EC 2008) has already incorporated biomarker responses (e.g. cytochrome-P450 quantification, lysosomal stability, metallothioneins) in various fish species (e.g. *Limanda limanda*, *Platichthys flesus*, *Hippoglossoides platessoides*) as early-warning signals of potential impacts at higher levels of organisation. Given the interrelation between the MSFD and the Water Framework Directive (WFW; EC 2000), the use of similar monitoring methodologies is under discussion and should be promoted in the latter directive. Overall, integrated results showed that, in addition to the macroinvertebrate community-based approach, biomarkers appear to be especially useful for providing appropriate information for diagnosing and characterising the impaired health status elicited by the overall exposure to chemical and other environmental stressors, functioning as rapid and cost-effective tools capable of early indicating ecosystem disruptions. The two approaches therefore

complement each other, reinforcing the need to use them in a combined way in order to achieve the best evaluation of ecosystems health and provide timely information improving recovery or mitigation interventions whenever necessary. The current study sets the foundations for future cost-effective biomonitoring campaigns in the rivers studied, since historical data is important to understand the ecosystems evolution and establishes baseline levels for the analysed biomarkers and macroinvertebrate taxa.

6.2. References

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