Ecological Indicators 145 (2022) 109645

Contents lists available at ScienceDirect

Ecological Indicators

journal homepage: www.elsevier.com/locate/ecolind

Original Articles

Ecotoxicological tools in support of the aims of the European Water Framework Directive: A step towards a more holistic ecosystem-based approach

Monica Martinez-Haro[a,b,*], Pelayo Acevedo[c], Antónia Juliana Pais-Costa[a], João M. Neto[a], Luis R. Vieira[d,e], Natalia Ospina-Alvarez[f], Mark A. Taggart[g], Lúcia Guilhermino[d,e], Rui Ribeiro[h], João Carlos Marques[a]

[a] MARE – Marine and Environmental Sciences Centre, Department of Life Sciences, University of Coimbra, Coimbra, Portugal
[b] Instituto Regional de Investigación y Desarrollo Agroalimentario y Forestal de Castilla-La Mancha (IRIAF), CIAG El Chaparrillo, Ciudad Real, Spain
[c] Instituto de Investigación en Recursos Cinegéticos, IREC (UCLM-CSIC-JCCM), Ciudad Real, Spain
[d] CIIMAR – Interdisciplinary Centre of Marine and Environmental Research of the University of Porto, Research Team of Ecotoxicology, Stress Ecology and Environmental Health (ECOTOX), Matosinhos, Portugal
[e] ICBAS – School of Medicine and Biomedical Sciences, University of Porto, Department of Population Studies, Laboratory of Ecotoxicology and Ecology (ECOTOX), Porto, Portugal
[f] Atlantic International Research Centre (AIR Centre), Terceira Island, Azores, Portugal
[g] Environmental Research Institute, University of the Highlands and Islands, Thurso, Scotland, UK
[h] Centre for Functional Ecology, Department of Life Sciences, University of Coimbra, Coimbra, Portugal

A R T I C L E I N F O

Keywords:
Estuarine invertebrates
Sediment toxicity
Sub-lethal endpoint
Environmental monitoring
Ecological quality assessment

A B S T R A C T

The Water Framework Directive (WFD) aims to attain 'good quality' status for all European water bodies through the achievement of good ecological status. To this purpose, the WFD advocates the creation of cost-effective monitoring tools to deliver appropriate data that help to create links between chemical and ecological indicators, as those from ecotoxicological research. Here, it was evaluated whether the integration of ecotoxicological tools, as bioassays and biomarkers, did (or did not) strengthen the robustness of the assessment of the ecological status obtained through well stablished biotic indices in two Atlantic estuaries. For that, a battery of *in-situ* bioassays, including five macroinvertebrate species (the crab *Carcinus maenas*, the amphipod *Echinogammarus marinus*, the isopod *Cyathura carinata*, the snail *Peringia ulvae* and the polychaete *Hediste diversicolor*, each one providing complementary information regarding key ecological functions) and a set of biomarkers, was used. In addition, the concentrations of heavy metals in sediments, and in macroalgae (*Fucus*), were determined, along with sediment granulometric, water physicochemical and nutrients characterization. We show that by interpreting the values of all indicators together, along with environmental components, it is possible to perform a more holistic description of the quality status of a waterbody – and, to begin to allude to factors limiting that quality. Ecotoxicological tools (*in situ* bioassays and biomarkers) appear to provide an added value of useful information for monitoring programmes regarding the true state - within which, both known and unknown contaminants are potentially present at concentrations sufficient to cause biological effects. Our results support the use of *in situ* bioassays and biomarkers within protocols aimed at fulfilling the goals of the WFD. In doing so, more complete and informative assessments of the ecological quality status of water bodies can take place.

1. Introduction

The Water Framework Directive (2000/60/EC, hereafter WFD) is the central piece of water quality legislation within Europe. One of its aims is to prevent further deterioration of European (EU) water resources and to protect and enhance the status of the water bodies, in terms of their ecosystem structure and/or function. The WFD pursues the sustainable management of water resources, whilst taking in account

* Corresponding author.
E-mail address: monica.martinez@uclm.es (M. Martinez-Haro).

https://doi.org/10.1016/j.ecolind.2022.109645
Received 28 June 2022; Received in revised form 2 November 2022; Accepted 4 November 2022
Available online 9 November 2022

environmental, economic and social dimensions. The 'Ecosystem Approach' inherent within the WFD is a reflection of Europe's increasing efforts to preserve the ecological integrity of the aquatic ecosystems, which is also in line with the aims of other European Directives (e.g., Habitats and Wild Fauna and Flora Directive – 1992/43/EEC and the Marine Strategy Framework Directive – 2008/56/EC).

A key goal of the WFD is to attain 'good quality' status for all EU water bodies – as assessed through chemical and biological quality elements. The chemical and ecological evaluations required by the WFD do however have limitations – i.e., there is not an explicit requirement to establish cause-effect relationships during the assessment of quality status (Allan et al., 2006a,b). Likewise, attaining and implementing a widely applicable, holistic and integrated assessment approach (as essentially required by the WFD) is very challenging (Hering et al., 2010; Beiras, 2016; Voulvoulis et al., 2017; Carvalho et al., 2019). Thus, in order to assess (and thus help achieve) 'good ecological status' for waterbodies, the WFD advocates the creation of new cost-effective monitoring tools that will deliver appropriate data – i.e., those that help create links between chemical and ecological status (Dworak et al., 2005; European Commission, 2010; Graveline et al., 2010). With the WFD as a driver, there is now the opportunity to start to take advantage of ecotoxicological research - and thus to create and integrate new cost-effective and cost-efficient monitoring toolboxes (Maas and van den Heuvel-Greve, 2004; Dworak et al., 2005; Allan et al., 2006b; de Jonge et al., 2006; Benedetti et al., 2012).

WFD guidance documents (European Commission, 2010) specifically suggest the use of ecotoxicological research in a triad approach. This approach concept was defined in the 1980s by Long and Chapman (1985) for sediments, and it is based on the integration of chemical (to measure contamination), bioassay (to measure toxicity) and infauna measurements (to measure community alterations), in order to link chemical and biological states under an ecological perspective (Chapman, 1990). Bioassays and biomarkers, as measures of toxicity, have been identified as ecotoxicological useful early warning tools facilitating a better understanding of cause–effect relationships, providing a more comprehensive evaluation of ecosystem and community health (Solimini et al., 2009; European Commission, 2010; Martinez-Haro et al., 2015; Wernersson et al., 2015; Vieira et al., 2018; Rodrigues et al., 2019, 2021; Lomartire et al., 2021; Santos et al., 2021; Schuijt et al., 2021). Together, bioassays and biomarkers have been recognised to provide useful information that cannot be obtained simply from the measurement of chemical residues nor biological samples. As tools, they have the capacity to respond to both known and unknown stressors which could cause dysfunction in a test organism (European Commission, 2010). Even though, such an approach is specifically recommended for investigative monitoring programmes (European Commission, 2003, 2009, 2010, 2014), many authors have noted the value of ecotoxicological tools within all three types of monitoring programme covered by the Directive (Mass and van den Heuvel-Greve, 2004; Allan et al., 2006a; Hamers et al., 2013; Wernersson et al., 2015). In this context, we aimed to assess whether the integration of ecotoxicological tools, as bioassays and biomarkers, did (or did not) strengthen the robustness of the assessment of the ecological status obtained through well stablished biotic indices. Ecologically relevant *in-situ* bioassays were used (i.e., a battery of five representative key macroinvertebrate estuarine species) to assess several key functions within six sites from two Atlantic estuaries. A set of biomarkers was then determined in the individuals used in *in situ* bioassays. In addition, metal contamination (total and labile fractions) was also studied in sediments from the monitoring sites, and in the algae *Fucus*. Our working hypothesis was that only addressing multiple indicators, the effect of pollution in the ecosystem would be detected and characterized more appropriately. Also, that the more holistic approach would help link cause and effect – and thus, be of greater value when designing control strategies.

2. Materials and methods

2.1. Study area

Six sampling sites in two estuaries located in the Northwest of the Iberian Peninsula, along the Portuguese coast, were selected in order to represent varying water and ecological quality status (Fig. 1). The estuaries were Lima (Viana do Castelo) and Mondego (Figueira da Foz), both part of the Ramsar Convention.

The Lima estuary generally receives anthropogenic pollution. In part, related to shipyards, commercial seaport operations, commercial fishing, marina activities, and to dredging of the navigational channel. But also, due to agricultural runoff, paper mill effluent and urban and industrial sewage (Costa-Dias et al., 2010). Moderate levels of pollution, compared with other estuaries, have been described previously (Guimarães et al., 2012; Azevedo et al., 2013).

The Mondego estuary is surrounded by a substantial human population, some industrial activity, saltpans, aquaculture, and also has a commercial and fishing harbour. The main pollution sources are from domestic and industrial sewage treatment plants and agriculture, namely from rice fields. Previous studies have described a high nutrient load in the estuary (Marques et al., 1993; Neto et al., 2008), but very low levels of heavy metals and organic contaminants (Pereira et al., 2005; Mil-Homens et al., 2014). However, elevated concentrations of endocrine disrupting compounds (Rocha et al., 2014), and pesticides residues above the maximum values established by European Directives were detected (Cruzeiro et al., 2016).

Three sampling sites were selected in each estuary (Fig. 1); the rational for their selection was their previous use in monitoring programs and availability of results regarding their ecological status (Table 1). This approach allowed us to work along a wide gradient of expected levels of pollution.

2.2. Sampling

The study was conducted in September 2013. From each site, water surface samples were collected for physicochemical parameters (temperature (°C), salinity, dissolve oxygen (%), and pH using YSI Professional Plus handheld multiparameter probe), and also for chlorophyll *a* total suspended solids (TSS), particulate organic matter (POM) and dissolved nutrient (mg/l; N-NO3, N-NO2, N-NH4, P-PO4 and silica). Three samples of sediment were randomly collected using a van Veen LMG grab with a 0.08 m^2 surface area. Samples of water were immediately filtered (Whatman GF/C glass fiber filter – 47 mm diameter, 1.2 m pore) and refrigerated in the dark until analysis. For TSS, the filter used (previously pre-weighed), was dried (60 ºC until constant weight), re-weighed, and the suspended material content was estimated as the weight difference (dry weight). POM was determined by weighing the same filter after combustion (450 ºC, 8 h) (ash weight). Chlorophyll *a* (μg/l) was quantified following Strickland and Parsons (1972) method. Nutrients were analysed by colorimetric reaction using a Skalar San ++® Continuous Flow AutoAnalyzer (Skalar, Germany; Strickland and Parsons,1972). Samples of sediments were immediately sieved using a 0.5 mm mesh (retaining that fraction greater than 0.5 mm) and fixed with 4 % buffered formalin solution. Once in the laboratory, samples were washed with tap water and sieved through a 1 mm and 0.5 mm sieve set. The retained organisms were sorted, counted and identified to species level (or the lowest taxonomic level possible) for biotic indices characterization (see below), taxonomy was standardised using the World Register of Marine Species (WoRMS, https://www.marinespecies.org). Biomass was estimated in terms of ash free dry weight (AFDW), after drying organisms at 60 °C until weight stabilisation and then obtaining the loss on ignition at 450 °C for 8 h.

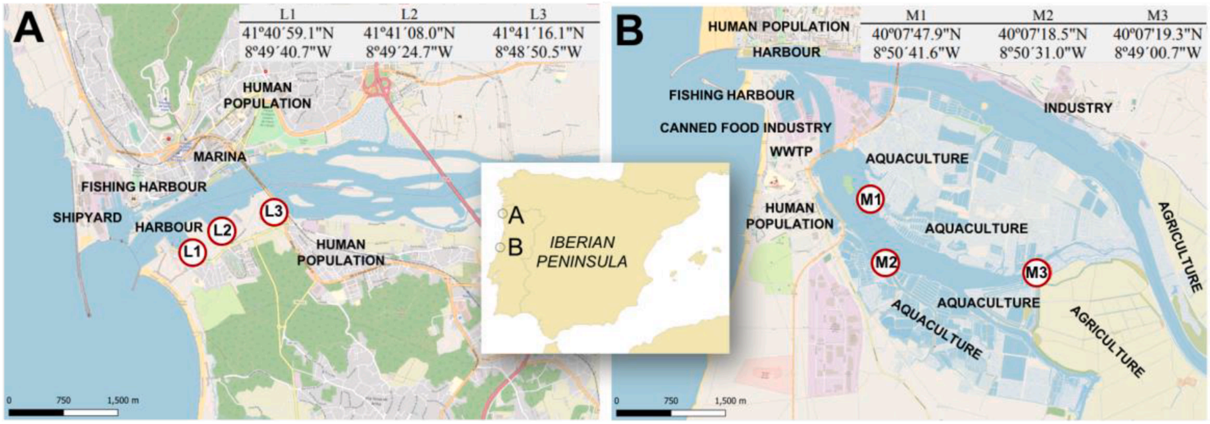

Fig. 1. Sampling sites at Lima and Mondego estuaries, and the main sources of pollution surrounding the sampling sites. WWTP refers to Wastewater Treatment Plant.

Table 1
Ecological status for the six sampling sites at the Lima (L1-L3) and Mondego (M1-M3) estuaries (sites coded from the mouth = 1 to upstream = 3). In brackets the code used in the referenced study.

Biotic Index	Lima L1	Lima L2	Lima L3	Mondego M1	Mondego M2	Mondego M3	Reference
AMBI	4.1	4.3	5.2				Azevedo et al., 2013 (sampling 2006-2007 Dec, Feb, May)
EQS	0.7		0.6				
BAT				0.52 (ST4)	0.84 (ST5)	0.78 (ST7)	Neto et al., 2010 (sampling spring 2006)
Bad	Poor	Moderate	Good	High			

AMBI: Azti's Marine Biotic Index; EQS: Ecological Quality Status; BAT: Portuguese-Benthic Assessment Tool.

2.3. Biotic indices

Abundance and biomass data were considered as individuals/m^2 and AFDW/m^2, respectively. The proportion of taxa, density and biomass in the community were calculated per sample and later for site. With these data, the number of species (s), the Shannon–Wiener (H), and the Margalef indices (d) were calculated. Then, the biotic indices AMBI (Borja et al., 2000), and BAT (Teixeira et al., 2009) were obtained for each of the sites (see further details in **Supplementary material**, **Table S1**).

2.4. Ecotoxicological tools

2.4.1. In-situ bioassays

A battery of five 48-h *in-situ* bioassays, based on postexposure feeding, was applied simultaneously at the six sites at the same time as the biological and environmental sampling took place (see further details about organisms harvesting and deployments in **Supplementary material**, **Figure S1**, **Table S2-S4**). The battery was composed of five key estuarine species: the crab *Carcinus maenas*, the amphipod *Echinogammarus marinus*, the isopod *Cyathura carinata*, the snail *Peringia ulvae* and the polychaete *Hediste diversicolor*. *Carcinus maenas* is an omnivorous feeder, consuming a large variety of preys (Cohen et al., 1995; Grosholz and Ruiz, 1996). This species has also been described to regulate community structure through predation and sediment disturbance during foraging (Jensen and Jensen, 1985; Matheson et al., 2016). *Echinogammarus marinus* is omnivorous, feeding on a wide range of plant material (including a number of algae species), as well as hard-bodied isopods and soft-bodied oligochaetes (Alexander et al., 2013). *Cyathura carinata* is an omnivorous species feeding on macroalgae (e.g. *Enteromorpha*), small invertebrates such as juvenile polychaetes, nematodes, oligochaetes, turbellarians, and detritus (Ólafsson and Persson, 1986). *Hediste diversicolor* is an omnivorous and opportunistic species, using different feeding modes such as filter-feeding, deposit-feeding, scavenging on the sediment surface for organic material and detritus, and predation (Scaps, 2002). Finally, *P. ulvae* has been described as a plastic species consuming both microphytobenthos and detritus (Riera, 2010). These benthic and epibenthic species inhabit soft and/or hard and seagrass meadows intertidal shallow estuarine and coastal habitats (Riera, 2010). These are abundant and widespread distributed species, covering the Atlantic coast, and Baltic and Mediterranean seas (Lincoln, 1979; Graham, 1988; Marques et al., 1994; Reish and Gerlinger, 1997; Kirkim et al., 2010), except *C. maenas*, which is distributed all over the world due to its invasive success (Carlton and Cohen, 2003; Hidalgo et al., 2005). These are prey of other crustacean species, fish and birds (Jensen and Jensen 1985; McLusky, 1989; Dumas and Witman, 1993; Hampel et al. 2005; Santos et al., 2005; Pasquaud et al., 2010;Martins et al., 2013).

2.4.2. Biomarker analysis

The levels of lipid peroxidation (LPO) and the activity of the enzymes cholinesterase (ChE), glutathione *S*-transferases (GST), and catalase (CAT) were determined in animals used in the *in situ* bioassays. *C. maenas* gills were separated for the determination of CAT activity. For *H. diversicolor*, *C. carinata* and *E. marinus*, heads were used for ChE determination. All the other biomarkers (GST in these 3 species, CAT in *H. diversicolor* and LPO in both *H. diversicolor* and *E. marinuss*) were determined using the remaining part of the body. Selected biomarkers for *C. carinata* and *E. marinus* were performed using 4 pools, of 3 organisms each one, from the same site. For *P. ulvae*, the specimen's body was carefully removed from the shell and homogenized for LPO. In this case, 9 pools of 9 organisms each one, from the same site were used for the biomarker testing. Tissues were isolated on ice and kept at 4 °C during all sample preparation and biomarker determination performed at 25 °C, as was described in Vieira et al. (2009) (further details of the procedures are included in the **Supplementary Material**).

2.5. Metal analyses

Natural process and human activities can redistribute heavy metals, which may greatly enhance its concentrations in land, water and also in the atmosphere. Adverse biological effects due to metal exposure has been described to increase with concentrations (Long et al., 1995), for which metal composition can inform either past and/or present sources of pollution, as well as the potential effect on organism inhabit the studied environment. Here, the total concentration of Cr, Mn, Ni, Cu, Zn, Cd, Pb and in surface sediments and *Fucus*, along with its labile fraction (i.e., free/easily dissociable ions, or, "bioavailable" fraction) in surface sediments was determined (see further details in **Supplementary material**, **Table S5**).

2.6. Statistical analysis

Multivariate methods (along with best professional judgment about the quality, extent, and congruence of data) were used to characterize the sites through the integration of nine different indicators and environmental parameters (Table 2). Principal Component Analysis (PCA) were performed on nine different groups of descriptors, corresponding to the biotic indicators, ecotoxicological tools and environmental components selected. Previously, the variables were auto-scaled (varimax normalized rotation) so they were treated with equal importance. Data were rearranged into a correlation matrix, which included the new variables extracted when considering eigenvalues higher than 1.0 (Kaiser's criteria). All analyses were performed using the Principal Component option of the Factorial Analysis procedure using SPSS Statistics 20.0.0.

3. Results and discussion

The descriptive statistics obtained from each of the indicators and environmental parameters studied are shown in **Supplementary material (Tables S6-S10)**. The data analysis performed for each of the study sites included a total of 64 variables (Table 3). After PCA analyses, 19 factors were defined (Fig. 2). Overall, results showed some dissimilarities in ecological status based on the classification performed when considering the biotic indices and ecotoxicological tools, suggesting the complementarity of both approaches. Hereafter, we would like to point out that the scores reported in Fig. 2 for a given factor and sampling site are always relative to the rest of sites according to the statistical design. Scores are therefore not interpretable in absolute terms but in relative ones and then always interpreted under a comparative perspective among sampling sites.

The selected sites in the Lima estuary were previously studied by Azevedo et al. (2013) in 2006–2007, who classified the sites L1 and L2 with moderate quality, and L3 with poor quality based on the biotic index AMBI. In that study, many other biological and environmental variables were considered and integrated as an index of relative ecological quality, which ranked the studied sites L1 and L2 as the least impacted, and L3 as the most impacted. In our study, we found that the sites L1 and L2 showed a similar pattern. From an ecological point of view, these sites showed the highest scores for the first factor of biotic indices, E1, which was positively related to the multivariate index BAT, s, H and d (Fig. 2, Table 3), indicating good quality status. Furthermore, these sites showed the highest scores for the first factor of *in-situ* bioassays, InSB1 (related to high feeding rates of *H. diversicolore* and *P. ulvae*, sediment-bound species), and the lowest scores for the second factor of biomarkers, BK2 (indicating low lipid peroxidation levels in *C. maenas*), suggesting that these sites are subject to low exposure to contaminants inducing oxidative stress or that they were able to deal with the stress avoiding lipid oxidative damage. Here, both biotic and ecotoxicological indices appear to agree, drawing a low pollution scenario in which a good quality status prevails. In terms of heavy metals, these sites showed intermediate scores for the first factor of metal concentrations in sediment (MS1), and *Fucus* (MF1), but the highest scores for the second factors (MS2 and MF2), related to levels of Mn in sediment, and of Cd and Zn in *Fucus,* respectively (Fig. 2, Table 3). Despite that, low-intermediate scores related to the labile metal fraction in sediments (LMS1 and LMS2) were detected, which suggest that the heavy metals had limited bioavailability, which reduced risk to local biota. Finally, regarding the environmental parameters, both sites showed the highest scores for the first factor related to sediment granulometry (SG1), indicating the presence of coarse sediment, high scores for the second factor of physicochemical parameters (PQ2) suggesting good water oxygenation, and intermediate scores for both factors related to nutrients. In general, our results draw a better ecological scenario to that described by Azevedo et al. (2013), where metal pollution seems to have been effectively managed.

In the case of the site L3, Azevedo et al. (2013) showed low metal levels in sediment, but, high nutrient, organic matter levels and faecal contamination in the water column, placing it as the most impacted site among those studied within the Lima estuary. Hence, this site was classified as heavily disturbed and of poor status (Azevedo et al., 2013). These authors highlighted the confined nature of the site and that it receives untreated wastewater. The results obtained here for L3 showed the highest score for the second factor related to the biotic indices (E2), but intermediate score for the first factor (E1), jointly suggesting moderate ecological status (Fig. 2). For *in-situ* bioassays, this site showed the lowest score for the first factor (InSB1), related to feeding response for

Table 2
Measurements included in each of the indicators (Biotic and ecotoxicological), and environmental components (sediment, *Fucus* and water) used to characterize the ecological conditions of different sites along the Lima and Mondego estuaries.

Biotic indices	**In-situ bioassays (feeding response)**	**Biomarkers (in *in-situ* organisms)**	**Heavy metals in sediment**	**Labile fraction of heavy metals in sediment**	**Heavy metals in *Fucus***	**Sediment granulometry**	Water physico-chemical	Water Nutrients
Azti Marine Biotic Index	*Carcinus*	*Cholinesterase*	Cd	Cd	Cd	Organic matter	Salinity	Si
Benthic Assessment Tool	*Echinogammarus*	*Glutathione S-Transferase*	Cr	Cr	Cr	<63	Temperature	NO_3
Number of species	*Cyathura*	*Catalase*	Cu	Cu	Cu	63-125	pH	NO_2
Shannon–Wiener index	*Hediste*	*Lipid peroxidation*	Mn	Mn	Mn	125-250	Oxygen (%)	PO_4
Margalef index	*Peringia*		Ni	Ni	Ni	250-500	Chlorophyll *a*	NH_3-N
			Pb	Pb	Pb	500-1000	Total suspended solids	
			Zn		Zn	1000-2000	Particulate organic matter	
						>2000		

Table 3
Summary of the scores obtained for the factors that emerged after Principal Component Analysis for the different indicators and environmental parameters analysed. The percentage of variance explained for each factor is shown in brackets.

Ecological indices	**E1 (59)**	**E2 (23)**	
BAT	**0.920**	-0.121	
AMBI	-0.008	**0.989**	
s	**0.782**	0.044	
H	**0.742**	0.406	
d	**0.957**	0.007	
Biomarkers	**BK1 (67)**	**BK2 (21)**	
Carcinus ChE	**-0.846**	-0.500	
Carcinus GST	**0.965**	0.151	
Carcinus CAT	**0.842**	0.384	
Carcinus LPO	-0.014	**0.990**	
Echinogammarus ChE	**-0.824**	-0.533	
Echinogammarus GST	**0.824**	0.104	
Echinogammarus LPO	**0.821**	0.497	
Cyathura ChE	**-0.889**	-0.128	
Cyathura GST	**0.730**	0.632	
Hediste ChE	**-0.880**	-0.285	
Hediste GST	**0.748**	0.605	
Hediste CAT	**0.942**	0.075	
Hediste LPO	**0.922**	0.247	
Peringia LPO	**0.804**	0.290	
Heavy metals in *Fucus*	**MF1 (40)**	**MF2 (36)**	**MF3 (18)**
Pb	0.029	0.048	**0.967**
Ni	**0.892**	0.090	0.222
Mn	**0.781**	-0.436	-0.395
Cd	0.081	**0.990**	0.067
Zn	-0.166	**0.978**	0.074
Cu	**0.956**	-0.037	-0.076
Cr	**0.660**	-0.619	0.349
Physico-chemical parameters of water	PQ1 (65)	PQ2 (18)	
Salinity	**0.829**	0.096	
Temperatura	**0.871**	-0.332	
pH	**-0.761**	0.036	
O2(%)	-0.069	**0.945**	
Chla	**0.895**	0.372	
SST	**0.890**	-0.220	
POMs	**0.954**	-0.203	

In-situ bioassay (feeding response)	**InSB1 (38)**	**InSB2 (37)**
Carcinus	0.568	**-0.768**
Echinogammarus	0.233	**0.603**
Cyathura	-0.218	**0.868**
Hediste	**0.833**	-0.336
Peringia	**0.881**	0.179
Heavy metals in sediment	**MS1 (78)**	**MS2 (16)**
Pb	**0.960**	-0.247
Ni	**0.927**	-0.093
Mn	-0.041	**0.993**
Cd	**0.940**	0.099
Zn	**0.990**	-0.032
Cu	**0.968**	0.116
Cr	**0.954**	-0.183
Labile fraction of heavy metals in sediment	**LMS1 (42)**	**LMS2 (41)**
Pb	-0.292	**-0.789**
Ni	-0.454	**0.743**
Mn	**0.786**	0.533
Cd	**0.977**	-0.133
Cu	**0.759**	0.315
Cr	0.328	**0.942**
Sediment granulometry	**GS1 (52)**	**GS2 (33)**
OM	-0.313	**0.945**
<63	-0.035	**0.969**
63-125	**-0.747**	0.523
125-250	**-0.920**	-0.369
250-500	**0.764**	-0.138
500-1000	**0.872**	-0.421
1000-2000	**0.856**	-0.365
>2000	**0.767**	-0.170
Nutrients in water	N1 (56)	N2 (31)
Si	**-0.836**	-0.115
NO_3	**0.841**	-0.062
NO_2	-0.611	**0.789**
PO_4	**0.960**	-0.001
NH_3-N	0.289	**0.949**

Hediste and *Peringia,* suggesting a sub-optimal status of the sediment. That was confirmed by the metal content. This site had the highest score for the first factor of heavy metals in sediments (MS1), reflecting high levels of Pb, Ni, Cd, Zn, Cu, and Cr (Fig. 2, Table 3), and the second highest score for the second factor (MS2) related to Mn concentration. In this sense, the maximum levels (also the minimum for Pb, Cu and Cr), of all these heavy metals exceeded the lowest effect levels previously reported for sediment (Burton, 2002; **Supplementary material, Table S9**). Despite this, the labile fraction of heavy metals in sediments appear to be low, except for Pb, reflecting by the low value of the second factor (LMS2, which was negatively related to Pb). Just Pb appeared to be especially high in *Fucus* (with the highest value for the third factor, MF3; Fig. 2). Regarding sediment granulometry, this site had the second highest score for the second factor (GS2), indicating a high presence of fine sediment and organic matter. In relation to water column parameters, this site showed the lowest scores of physicochemical parameters (PQ1, PQ2), and the second highest for the first factor related to nutrients (N1) reaffirming the confined nature, and the nutrient load described by Azevedo et al. (2013) for this site. Overall, our results showed that both biotic and ecotoxicological indices analysed seem to agree, as in the other two sites analysed, but this time drawing a scenario of pollution, even worse to the one described by Azevedo et al. (2013).

Concerning the Mondego estuary, the selected sites were previously studied by Neto et al. (2010). Regarding the site M1 (site ST4 in Neto et al, 2010), a decrease in ecological quality status at this site was detected from 1998 to 2006, suggesting that the macroalgal blooms occurred in

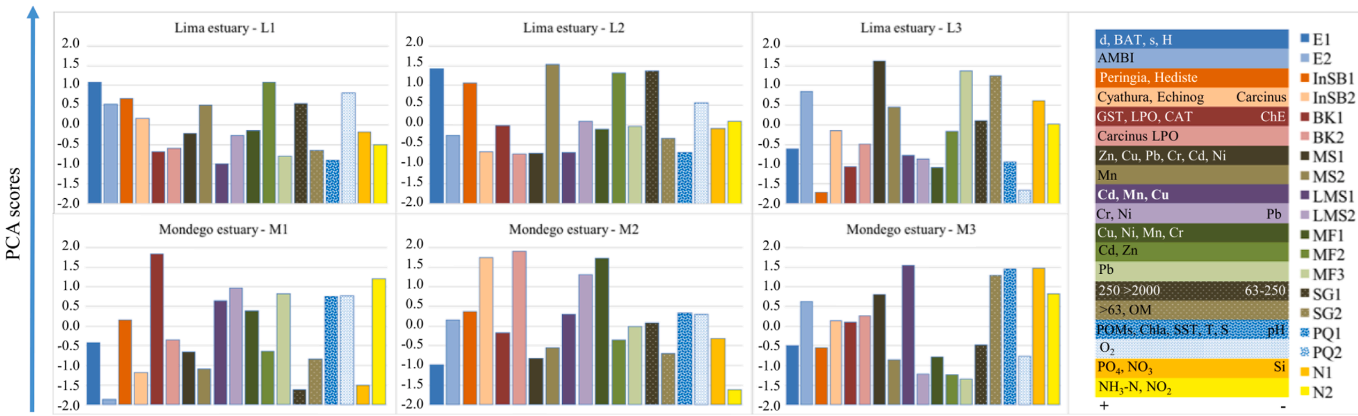

Fig. 2. Integration of the different indicators and environmental parameters used to assess the ecological status for sites in the Lima and Mondego estuaries. Biotic indices (E1 and E2), *in-situ* bioassay (based on feeding response, InSB1 and InSB2), biomarkers (measured on the individuals used in the *in-situ* bioassays; BK1 and BK2), heavy metals in sediment (MS1 and MS2), its labile fraction (LMS1 and LMS2), heavy metals in *Fucus* (MF1, MF2 and MF3), sediment granulometry (SG and SG2), and physicochemical parameters (PQ1 and PQ2) and nutrients in water (N1 and N2). Scores regarding the PCA factors (after varimax normalized rotation) for each sampling site are represented.

1998 had perturbed the macroinvertebrate community since that time. Results obtained here showed the lowest score for the second factor of the biotic indices (E2), indicating high ecological status according to AMBI, but a medium score for E1, for which a moderate status emerged based on d, BAT, s and H (Fig. 2). When considering the *in-situ* bioassay and biomarker results, this site showed intermediate scores for both factors related to the *in situ* bioassays (InSB1 and InSB2), and the highest score for the first factor of biomarkers (BK1), indicating inhibition of ChE activity, induction of GST and CAT activities, and enhanced LPO levels (**Supplementary material**, **Table S8**), suggesting that the ecological community at this site was subject to stress conditions. Enhanced GST and CAT activities, along with increased lipid peroxidation have been related to oxidative stress conditions in *C. maenas* (Elumalai et al., 2007) and other aquatic invertebrates (Barata et al., 2005). Inhibition of ChE activity can occur through exposure to heavy metals, detergents and even microplastics (Guilhermino et al., 1998; Elumalai et al., 2002; Frasco et al., 2005; Richetti et al., 2011; Barboza et al., 2018). However, this enzyme is very sensitive to anticholinesterase insecticides (namely, organophosphate and carbamate insecticides), designed specifically to act directly on this enzyme (Ecobichon, 2001). These pesticides are widely used in agriculture. In this sense, the inhibition of ChE activity could be explained by the fact that the drainage basin of the Mondego river encompasses 667,000 ha, including an important area dedicated to agriculture (12,300 ha; Ferreira dos Santos and Freitas, 2012), which is especially intense downstream (Fig. 1). Agriculture, mostly involved maize, rice and potato crops, from which, residues of herbicides and insecticides have previously been detected in surface water, including several organophosphate and carbamate insecticides (Andrade and Stigter, 2009; Silva et al., 2015; Cruzeiro et al., 2016; Silva et al., 2015). Looking at heavy metals, despite the low levels detected in sediments, defined by the low scores of the factors MS1 and MS2, these showed high labile fraction values, as was indicated by the elevated scores for the factors LMS1 and LMS2 (positively related to Cd, Mn, Cu, Cr and Ni; Fig. 2), and the especially high levels of Pb detected in *Fucus.* Such exposure to heavy metal could also contribute to the stress scenario captured by biomarkers, as heavy metals are known to tend to enhance oxidative stress in organisms (Ercal et al., 2001). Finally, noted that water column parameters analysed also captured a stressful scenario, especially thought the first and second factors of the physicochemical (PQ1) and nutrients (N2) parameters, respectively, related to high POM, TSS, Chl*a*, PO4 and NO3 content, which could again be related to agriculture, but also to aquaculture activities downstream (Fig. 1).

Overall, our results show discrepancies between the information provided by the biotic indices and ecotoxicological tools. Moreover, the biotic indices analysed seem to have difficulties in characterising the status of this site, being detected discrepancies between them. In contrast, the ecotoxicological tools analysed show a stressed system compatible with a high pollution scenario. Scenario also captured through the analysis of heavy metals and environmental parameters.

For the site M2, previous work had described a stable site with a good status since the early 1990s, and a high status in 2005 and 2006 (Neto et al., 2010, station ST5). In the current study, this site showed the lowest score for the first factor related to the biotic indices (E1), and intermediate score for the second factor (E2). Results showed a low score for the first factor related to *in-situ* bioassays (InSB1, suggesting feeding impairment for *Hediste* and *Peringia),* but the highest score for InSB2 (positively related to *Cyathura* and *Echinogammarus* and negatively to *Carcinus*) (Fig. 2, Table 3**)**. The high feeding response of *Cyathura* and *Echinogammarus* could relate to the fact that this can be one of the most optimum sites analysed for this species, due to it is one of the sites within the Mondego estuary where the highest population of both species have been registered (Marques and Nogueira, 1991; Marques et al., 1994; Ferreira et al., 2004; Leite et al., 2014). In this site, biomarkers showed the highest score for the second factor (BK2), which is directly related to lipid peroxidation levels in *C. maenas,* which in turn reflects the low feeding response detected in the *in-situ* bioassay. In fact, it is noteworthy that despite showing low level of pollution (which can be seen from the low scores on the factors related to heavy metals in sediments), this site had the highest score for the second factor of the labile metal fraction in sediments, LMS2, related to Ni and Cr concentrations. Furthermore, this site showed the highest score for the first factor regarding heavy metals in *Fucus* (MF1), indicating high levels of Cu, Ni, Mn and Cr (Fig. 2, Table 3). Regarding, sediment granulometry, and water column parameters, this site had intermediate-low values, without any remarkable variable. As observed at site M1, biotic indices analysed seem to show some disagreement. Moreover, the ecotoxicological tools analysed also suggest that this is a site with a higher complexity. Despite the low level of heavy metals detected in the sediments, they appear to be highly bioaccessible, as suggested by the high percentages of labile fraction as well as high levels in *Fucus,* drawing an overall stressed environment.

Finally, the site M3 showed intermediate-low score for the first factor related to biotic indices (E1), and intermediate-high score for the second factor (E2), for which a moderate/good status emerged. In contrast, *in-situ* bioassays showed intermediate-low feeding activities for all the species tested. Biomarkers also detected intermediate-low scores for the two factors (Fig. 2) suggesting lower physiological stress at this site than for sites M1 and M2, although higher than for sites in the Lima estuary.

Considering heavy metals in sediment, this site showed the second highest score for MS1, positively related to Zn, Cu, Pb, Cr, Cd and Ni and levels (Fig. 2, Table 3). In this sense, note that the levels of Pb, Ni and Cr exceed the lowest effect level previously reported for sediment (Burton, 2002; **Supplementary material**, **Table S9**). Furthermore, this site showed the highest score for the first factor related to labile heavy metals in sediments (LFMS1), and the lowest score for the second factor (indicating high bioavailability for Cd, Mn, Cu, and Pb; Fig. 2, Table 3). Despite of this, low scores for the three factors related to heavy metals in *Fucus* were detected (Fig. 2). This site showed the second highest score for the second factor of the sediment granulometry (SG2), related to fine particles (<63 μm) and organic matter. In fact, this site had the highest score for the first factor of physicochemical parameters, positively related to POM, Chl*a*, TSS. Additionally, this site had the highest score for the first factor of nutrients in water and the second highest of the second factor, positively related to nitrate, phosphate and ammonium levels. In previous work in the Mondego estuary, this site was considered to have a good status (since the late 90′s; Neto et al., 2010, station ST7), but our results suggest that perturbation has emerged, affecting the quality of this site. In fact, this site is surrounding by aquaculture and agricultural lands, more specifically rice fields (Fig. 1), commonly associated with nutrient discharges (Marques et al., 1993). In addition, agricultural lands are commonly subjected to pesticide treatment, which may contain residues of heavy metals such as As, Pb, Ni, Cu, Zn, Cr (Defarge et al., 2018; Seralini and Jungers, 2020), which could be one of the factors explaining the levels of such heavy metals detected at this site. Overall, on this site, biotic indices and ecotoxicological tools also showed some discrepancies. While the biotic indices analysed suggest that it is a site close to good quality, ecotoxicological tools, and environmental parameters show a stressed and more polluted scenario.

4. Conclusions

Overall, both biotic indices and ecotoxicological tools (such as biomarkers and *in-situ* bioassays) could thus be used in a complementary way, even when, in the first instance, a good ecological status appears to be present. We here provide its usefulness in order to obtain a more in-depth characterization of the water quality status. The combined use of biotic indices and toxicological tools can help us to overcome the limitations of both tools in capturing the complexity of these ecological systems. Ecotoxicological tools appear to provide useful information regarding the potentially presence of both known and unknown contaminants at concentrations sufficient to cause biological effects, for which its use in surveillance monitoring has been suggested previously for many authors (Maas and van den Heuvel-Greve, 2004; Allan et al., 2006a; Hamers et al., 2013). In fact, these have been included as supportive/interpretative parameters, in benthic invertebrate and fish biological elements for lakes, and in fish for transitional waters (European Commission, 2003). Recently, Brack et al. (2017), under the EU FP7 Collaborative Project SOLUTIONS and the European monitoring network NORMAN, made 10 recommendations to improve monitoring within the WFD. Among these, the implementation of *in situ* bioassays and biomarkers into water quality monitoring was highlighted as a key approach to aid in the consistent assessment of contamination (Di Paolo et al., 2016; Brack et al., 2017). Similar recommendations have emerged from other European projects such as SWIFT-WFD (Maas and van den Heuvel-Greve, 2004), ECOMAN (Galloway et al., 2006) or Team Minho (Beiras, 2016). Furthermore, Poikane et al. (2020), after reviewing more than 400 national ecological assessment methods for European water bodies, highlighted that current assessment methods do not reliably detect effects of toxic pollution, and that effect-based tools could be developed to better integrate the effects of different toxicants into biological assessments. Finally, as Maas and van den Heuvel-Greve (2004) noted, we suggest that although the integration of these tools may initially increase costs, the extra expenditure can easily be recovered by taking effective measures and anticipating effects on an ecological scale.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgment

We are grateful to all those who assisted during the field and laboratory work.

Funding sources.

This paper is a result of the project QWATER (Bioassay integration under the European Water Framework Directive: A step towards an ecological approach) funded by the 7th Framework Programme (FP7 2007-2013) of the European Commission through a Marie Curie Intra-European Fellowship for Career Development (PIEF-GA-2011-299747), https://qwaterprojecteu.wordpress.com.

JMN acknowledges the financial programme of the Fundação para a Ciência e Tecnologia, I. P (FCT) through the Individual support attributed in the scope of Decry-Law n° 57/2016, from 29th August. This study had the support of national funds through FCT and ERDF in the framework of the programme Portugal 2020, under the projects UIDB/04292/2020, UIDP/04292/2020, granted to MARE; LA/P/0069/2020, granted to the Associate Laboratory ARNET; UIDB/Multi/04423/2020 granted to CIIMAR; and LA/P/0101/2020 granted to CIMAR-LA.

Appendix A. Supplementary data

Supplementary material includes further description of the methods used and of the results obtained. Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2022.109645.

References

Alexander, M.E., Dick, J.T.A., O'Connor, N.E., 2013. Born to kill: predatory functional responses of the littoral amphipod Echinogammarus marinus Leach throughout its life history. J. Exp. Mar. Biol. Ecol. 439, 92–99.

Allan, I.J., Vrana, B., Greenwood, R., Mills, G.A., Knutsson, J., Holmberg, A., Guigues, N., Fouillac, A.M., Laschi, S., 2006a. Strategic monitoring for the European Water Framework Directive. TrAC - Trends Anal. Chem. 25, 704–715.

Allan, I.J., Vrana, B., Greenwood, R., Mills, G.A., Roig, B., Gonzalez, C., 2006b. A "toolbox" for biological and chemical monitoring requirements for the European Union's Water Framework Directive. Talanta 69, 302–322.

Andrade, A.I.A.S.S., Stigter, T.Y., 2009. Multi-method assessment of nitrate and pesticide contamination in shallow alluvial groundwater as a function of hydrogeological setting and land use. Agric. Water Manag. 96, 1751–1765.

Azevedo, I., Ramos, S., Mucha, A.P., Bordalo, A.A., 2013. Applicability of ecological assessment tools for management decision-making: A case study from the Lima estuary (NW Portugal). Ocean and Coast. Manag. 72, 54–63.

Barata, C., Lekumberri, I., Vila-Escalé, M., Prat, N., Porte, C., 2005. Trace metal concentration, antioxidant enzyme activities and susceptibility to oxidative stress in the trichoptera larvae Hydropsyche exocellata from the Llobregat river basin (NE Spain). Aquat. Toxicol. 74, 3–19.

Barboza, L.G.A., Vieira, L.R., Branco, V., Figueiredo, N., Calvalho, F., Calvalho, C., Guilhermino, L., 2018. Microplastics cause neurotoxicity, oxidative damage and energy-related changes and interact with the bioaccumulation of mercury in the European seabass, Dicentrarchus labrax (Linnaeus, 1758). Aquat. Toxicol. 195, 49–57.

Beiras, R., 2016. Assessing EcologicalStatus of Transitional and Coastal Waters; Current Difficulties and Alternative Approaches. Front. Mar. Sci 3, 88.

Benedetti, M., Ciaprini, F., Piva, F., Onorati, F., Fattorini, D., Notti, A., Ausili, A., Regoli, F., 2012. A multidisciplinary weight of evidence approach for classifying polluted sediments: Integrating sediment chemistry, bioavailability, biomarkers responses and bioassays. Environ. Int. 38, 17–28.

Borja, A., Franco, J., Pérez, V., 2000. A Marine Biotic Index to Establish the Ecological Quality of Soft-Bottom Benthos within European Estuarine and Coastal Environments. Mar. Pollut. Bull. 40, 1100–1114.

Brack, W., Dulio, V., Ågerstrand, M., Allan, I., Altenburger, R., Brinkmann, M., Bunke, D., Burgess, R.M., Cousins, I., Escher, B.I., Hernández, F.J., Hewitt, L.M., Hilscherová, K., Hollender, J., Hollert, H., Kase, R., Klauer, B., Lindim, C.,

Herráez, D.L., Miège, C., Munthe, J., O'Toole, S., Posthuma, L., Rüdel, H., Schäfer, R. B., Sengl, M., Smedes, F., van de Meent, D., van den Brink, P.J., van Gils, J., van Wezel, A.P., Vethaak, A.D., Vermeirssen, E., von der Ohe, P.C., Vrana, B., 2017. Towards the review of the European Union Water Framework Directive: recommendations for more efficient assessment and management of chemical contamination in European surface water resources. Sci. Total Environ. 576, 720–737.
Burton Jr, G.A., 2002. Sediment quality criteria in use around the world. Limnology 3, 65–75.
Carlton, J.T., Cohen, A.N., 2003. Episodic global dispersal in shallow water marine organisms: The case history of the European shore crabs Carcinus maenas and C. aestuarii. J. Biogeogr. 30, 1809–1820.
Carvalho, L., Mackay, E.B., Cardoso, A.C., Baattrup-Pedersen, A., Birk, S., Blackstock, K. L., Borics, G., Borja, A., Feld, C.K., Ferreira, M.T., Globevnik, L., Grizzetti, B., Hendry, S., Hering, D., Kelly, M., Langaas, S., Meissner, K., Panagopoulos, Y., Penning, E., Rouillard, J., Sabater, S., Schmedtje, U., Spears, B.M., Venohr, M., van de Bund, W., Solheim, A.L., 2019. Protecting and restoring Europe's waters: An analysis of the future development needs of the Water Framework Directive. Sci. Total Environ. 658, 1228–1238.
Chapman, P.M., 1990. The Sediment Quality Triad approach to determining pollution-induced degradation. Sci. Total Environ. 97–8, 815–825.
Cohen, A.N., Carlton, J.T., Fountain, M., 1995. Introduction, dispersal, and potential impacts of the green crab Carcinus maenas in San Francisco Bay. CA. Mar. Biol. 122, 225–237.
Costa-Dias, S., Sousa, R., Antunes, C., 2010. Ecological quality assessment of the lower Lima Estuary. Mar. Pollut. Bull. 61, 234–239.
Cruzeiro, C., Rocha, E., Pardal, M.A., Rocha, M.J., 2016. Environmental assessment of pesticides in the Mondego River Estuary (Portugal). Mar. Pollut. Bull. 103, 240–246.
de Jonge, V.N., Elliott, M., Brauer, V.S., 2006. Marine monitoring: Its shortcomings and mismatch with the EU Water Framework Directive's objectives. Mar. Pollut. Bull. 53, 5–19.
Defarge, N., Spiroux de Vendomois, J., Seralini, G.E., 2018. Toxicity of formulants and heavy metals in glyphosate-based herbicides and other pesticides. Toxicology Reports 5, 156–163.
Di Paolo, C., Ottermanns, R., Keiter, S., Ait-Aissa, S., Bluhm, K., Brack, W., Breitholtz, M., Buchinger, S., Carere, M., Chalon, C., Cousin, X., Dulio, V., Escher, B.I., Hamers, T., Hilscherova, K., Jarque, S., Jonas, A., Maillot-Marechal, E., Marneffe, Y., Nguyen, M. T., Pandard, P., Schifferli, A., Schulze, T., Seidensticker, S., Seiler, T.B., Tang, J., van der Oost, R., Vermeirssen, E., Zounkova, R., Zwart, N., Hollert, H., 2016. Bioassay battery interlaboratory investigation of emerging contaminants in spiked water extracts—towards the implementation of bioanalytical monitoring tools in water quality assessment and monitoring. Water Res. 104, 473–484.
Dumas, J.V., Witman, J.D., 1993. Predation by Herring Gulls (Larus argentatus Coues) on two rocky intertidal crab species [Carcinus maenas (L.) & Cancer irroratus Say]. J. Exp. Mar. Biol. Ecol. 169, 89–101.
Dworak, T., Gonzalez, C., Laaser, C., Interwies, E., 2005. The need for new monitoring tools to implement the WFD. Environ. Sci. Policy 8, 301–306.
Ecobichon, D.J., 2001. Toxic effects of pesticides. In: Klaassen, C.D. (Ed.), Casarett and Doull's Toxicology, 6th ed. McGraw-Hill Medical Publishing Division, Toronto, pp. 763–810.
Elumalai, M., Antunes, C., Guilhermino, L., 2002. Effects of single metals and their mixtures on selected enzymes of Carcinus Maenas. Water Air Soil Pollut. 141, 273–280.
Elumalai, M., Antunes, C., Guilhermino, L., 2007. Enzymatic biomarkers in the crab Carcinus maenas from the Minho River estuary (NW Portugal) exposed to zinc and mercury. Chemosphere 66, 1249–1255.
Ercal, N., Gurer-Orhan, H., Aykin-Burns, N., 2001. Toxic metals and oxidative stress part I: Mechanisms involved in metal oxidative damage. Curr. Top. Med. Chem. 1, 529–539.
European Commission, 2003. Directorate-General for Environment, Monitoring under the water framework directive. Guidance document N° 7, Publications Office.
European Commission, 2009. Directorate-General for Environment, Guidance on surface water chemical monitoring under the water framework directive. Guidance document N° 19, Publications Office. https://data.europa.eu/doi/10.2779/72701.
European Commission, 2010. Directorate-General for Environment, Guidance on chemical monitoring of sediment and biota under the water framework directive. Guidance document N° 25, Publications Office. https://data.europa.eu/doi/10.2779/43586.
European Commission, 2014. Directorate-General for Environment, Technical report on aquatic effect-based monitoring tools, Publications Office. https://data.europa.eu/doi/10.2779/72812.
Ferreira dos Santos, J., Freitas, V. 2012. Aproveitamento hidroagrícola do Baixo Mondego actualidade e desafios futuros. In: aph (Ed.), IV Congresso nacional de rega e drenagem. Direcção-Geral de Agricultura e Desenvolvimento Rural (DGADR), p. 14 Coimbra.
Ferreira, S.M., Pardal, M.A., Lillebø, A.I., Cardoso, P.G., Marques, J.C., 2004. Population dynamics of Cyathura carinata (Isopoda) in a eutrophic temperate estuary. Estuar. Coast. Shelf Sci. 61, 669–677.
Frasco, M.F., Fournier, D., Carvalho, F., Guilhermino, L., 2005. Do metals inhibit acetylcholinesterase (AchE)? Implementation of assay conditions for the use of AchE activity as a biomarker of metal toxicity. Biomarkers 10, 360–375.
Galloway, T.S., Brown, R.J., Browne, M.A., Dissanayake, A., Lowe, D., Depledge, M.H., Jones, M.B., 2006. The ECOMAN project: A novel approach to defining sustainable ecosystem function. Mar. Pollut. Bull. 53, 186–194.
Graham, A.F.R.S., 1988. Molluscs: Prosobranch and Pyramidellid Gastropods: Key and Notes for the Identification of the Species, 2nd ed. Linnean Society of London, London, UK.
Graveline, N., Maton, L., Rinaudo, J.D., Lückge, H., Interwies, E., Rouillard, J., Strosser, P., Palkaniete, K., Taverne, D., 2010. An operational perspective on potential uses and constraints of emerging tools for monitoring water quality. TrAC - Trends Anal. Chem. 29, 378–384.
Grosholz, E.D., Ruiz, G.M., 1996. Predicting the impact of introduced marine species: lessons from the multiple invasions of the European green crab Carcinus maenas. Biol. Conserv. 78, 59–66.
Guilhermino, L., Barros, P., Silva, M.C., Soares, A.M.V.M., 1998. Should the use of inhibition of cholinesterases as a specific biomarker for organophosphate and carbamate pesticides be questioned? Biomarkers 3, 157–163.
Guimarães, L., Medina, M.H., Guilhermino, L., 2012. Health status of Pomatoschistus microps populations in relation to pollution and natural stressors: Implications for ecological risk assessment. Biomarkers 17, 62–77.
Hamers, T., Legler, J., Blaha, L., Hylland, K., Marigomez, I., Schipper, C.A., et al., 2013. Expert opinion on toxicity profiling—report from a NORMAN expert group meeting. Integr. Environ. Assess. Manag. 9, 185–191.
Hampel, H., Cattrijsse, A., Elliott, M., 2005. Feeding habits of young predatory fishes in marsh creeks situated along the salinity gradient of the Schelde estuary, Belgium and The Netherlands. Helgol. Mar. Res. 59, 151–162.
Hering, D., Borja, A., Carstensen, J., Carvalho, L., Elliott, M., Feld, C.K., Heiskanen, A.-S., Johnson, R.K., Moe, J., Pont, D., Solheim, A.L., de Bund, W.V., 2010. The European Water Framework Directive at the age of 10: A critical review of the achievements with recommendations for the future. Sci. Total Environ. 408, 4007–4019.
Hidalgo, F.J., Baron, P.J., Orensanz, J.M., 2005. A prediction come true: The green crab invades the Patagonian coast. Biol. Invasions 7, 547–552.
Jensen, K.T., Jensen, J.N., 1985. The importance of some epibenthic predators on the density of juvenile benthic macrofauna in the Danish Wadden sea. J. Exp. Mar. Biol. Ecol. 89, 157–174.
Kirkim, F., Özcan, T., Katağan, T., Bakir, K., 2010. First record of five free-living isopod species from the coast of Cyprus. Acta Adriat. 51, 101–105.
Leite, N., Guerra, A., Almeida, A., Marques, J.C., Martins, I., 2014. Long term variation of an amphipod species' population secondary production as indicator of incomplete resilience in a temperate estuary. Ecol. Indic. 36, 324–333.
Lincoln, R.J., 1979. British Marine Amphipoda: Gammaridea. British Museum (Natural History), London.
Lomartire, S., Marques, J.C., Gonçalves, A.M.M., 2021. Biomarkers based tools to assess environmental and chemical stressors in aquatic systems. Ecol. Indic. 122, 107207.
Long, E.R., Chapman, P.M., 1985. A sediment quality triad: measures of sediment contamination, toxicity and infaunal community composition in Puget Sound. Mar. Pollut. Bull. 16, 405–415.
Long, E.R., MacDonald, D.D., Smith, S.L., Calder, F.D., 1995. Incidence of adverse biological effects within ranges of chemical concentrations in marine and estuarine sediments. Environ. Manage. 19, 81–97.
Maas, J.L., van den Heuvel-Greve, M.J., 2004. Opportunities for bio-analysis in WFD chemical monitoring using bioassays. RIZA working document 2005, 053X.
Marques, J.C., Nogueira, A., 1991. Life cycle, population dynamics and production of Echinogammarus marinus (Leach) (Amphipoda) in the Mondego estuary (Portugal). Oceanol. Acta 11, 213–223.
Marques, J.C., Maranhao, P., Pardal, M.A., 1993. Human impact assessment on the subtidal microbenthic community structure in the Mondego estuary (western Portugal). Estuar. Coast. Shelf Sci. 37, 403–419.
Marques, J.C., Martins, I., Teles-Ferreira, C., Cruz, S., 1994. Population dynamics, life history, and production of Cyathura carinata (Krøyer) (Isopoda: Anthuridae) in the Mondego Estuary. Portugal. J. Crust. Biol. 14, 258–272.
Martinez-Haro, M., Beiras, R., Bellas, J., Capela, R., Coelho, J.P., Lopes, I., Moreira-Santos, M., Reis-Henriques, A.M., Ribeiro, R., Santos, M.M., Marques, J.C., 2015. A review on the Ecological Quality Status assessment in aquatic systems using community based indicators and ecotoxicological tools: what might be the added value of their combination? Ecol. Indic 48, 8–16.
Martins, R.C., Catry, T., Santos, C.D., Palmeirim, J.M., Granadeiro, J.P., 2013. Seasonal variations in the diet and foraging behaviour of Dunlins Calidris alpina in a south European estuary: improved feeding conditions for northward migrants. PLoS ONE 8, e81174.
Matheson, K., McKenzie, C.H., Gregory, R.S., Robichaud, D.A., Bradbury, I.R., Snelgrove, P.V.R., Rose, G.A., 2016. Linking eelgrass decline and impacts on associated fish communities to European green crab Carcinus maenas invasion. Mar. Ecol. Prog. Ser. 548, 31–45.
McLusky, D., 1989. The estuarine ecosystem, 2nd edn. Blackie and Sons Ltd, London.
Mil-Homens, M., Vale, C., Raimundo, J., Pereira, P., Brito, P., Caetano, M., 2014. Major factors influencing the elemental composition of surface estuarine sediments: The case of 15 estuaries in Portugal. Mar. Pollut. Bull. 84, 135–146.
Neto, J.M., Flindt, M.R., Marques, J.C., Pardal, M.A., 2008. Modelling nutrient mass balance in a temperate meso-tidal estuary: implications for management. Estuar. Coast. Shelf Sci. 76, 175–185.
Neto, J.M., Teixeira, H., Patrício, J., Baeta, A., Veríssimo, H., Pinto, R., Marques, J.C., 2010. The response of estuarine macrobenthic communities to natural- and human-induced changes: dynamics and ecological quality. Estuar. Coast. 33, 1327–1339.
Ólafsson, E.B., Persson, L.E., 1986. Distribution, life cycle and demography in a brackish water population of the isopod Cyathura carinata (Krøyer) (Crustacea). Estuar. Coast. Shelf Sci. 23, 673–687.
Pasquaud, S., Pillet, M., David, V., Sautour, B., Elie, P., 2010. Determination of fish trophic levels in an estuarine system. Estuar. Coast. Shelf Sci. 86, 237–246.

Pereira, P., Vale, C., Ferreira, A.M., Pereira, E., Pardal, M.A., Marques, J.C., 2005. Seasonal variation of surface sediments composition in Mondego River Estuary. J. Environ. Sci. Health, Part A 40, 317–329.
Poikane, S., Salas, H.F., Kelly, M.G., Borja, A., Birk, S., van de Bund, W., 2020. European aquatic ecological assessment methods: A critical review of their sensitivity to key pressures. Sci. Total Environ. 740, 140075.
Reish, D.J., Gerlinger, T.V., 1997. A review of the toxicological studies with polychaetous annelids. Bull. Mar. Sci. 60, 584–607.
Richetti, S.K., Rosemberg, D.B., Ventura-Lima, J., Monserrat, J.M., Bogo, M.R., Bonan, C. D., 2011. Acetylcholinesterase activity and antioxidant capacity of zebrafish brain is altered by heavy metal exposure. Neurotoxicology 32, 116–122.
Riera, P., 2010. Trophic plasticity of the gastropod Hydrobia ulvae within an intertidal bay (Roscoff, France): a stable isotope evidence. J. Sea Res. 63, 78–83.
Rocha, M.J., Cruzeiro, C., Reis, M., Pardal, M.A., Rocha, E., 2014. Spatial and seasonal distribution of 17 endocrine disruptor compounds in an urban estuary (Mondego River, Portugal): evaluation of the estrogenic load of the area. Environ. Monit. Assess. 186, 3337–3350.
Rodrigues, C., Guimaraes, L., Vieira, N., 2019. Combining biomarker and community approaches using benthic macroinvertebrates can improve the assessment of the ecological status of rivers. Hydrobiologia 839, 1–24.
Rodrigues, S., Pinto, I., Martins, F., Formigo, N., Antunes, S.C., 2021. Can biochemical endpoints improve the sensitivity of the biomonitoring strategy using bioassays with standard species, for water quality evaluation? Ecotoxicol. Environ. Saf. 215, 112151.
Santos, C.D., Granadeiro, J.P., Palmeirim, J.M., 2005. Feeding ecology of Dunlin Calidris alpina in a southern European estuary. Ardeola 52, 235–252.
Santos, J.I., Vidal, T., Goncalves, F.J.M., Castro, B.B., Pereira, J.L., 2021. Challenges to water quality assessment in Europe – Is there scope for improvement of the current Water Framework Directive bioassessment scheme in rivers? Ecol. Indic. 121, 107030.
Scaps, P., 2002. A review of the biology, ecology and potential use of the common ragworm Hediste diversicolor (O.F. Müller) (Annelida: Polychaeta). Hydrobiologia 470, 203–218.
Schuijt, L.M., Peng, F.-J., van den Berg, S.J.P., Dingemans, M.M.L., Van den Brink, P.J., 2021. (Eco)toxicological tests for assessing impacts of chemical stress to aquatic ecosystems: Facts, challenges, and future. Sci. Total Environ. 795, 148776.
Seralini, G.E., Jungers, G., 2020. Toxic compounds in herbicides without glyphosate. Food Chem. Toxicol. 146, 111770.
Silva, E., Dam, M.A., Cerejeira, M.J., 2015. Aquatic risk assessment of priority and other river basin specific pesticides in surface waters of Mediterranean river basins. Chemosphere 135, 394–402.
Solimini, A.G., Ptacnik, R., Cardoso, A.C., 2009. Towards holistic assessment of the functioning of ecosystems under the Water Framework Directive. TrAC - Trends Anal. Chem. 28, 143–149.
Strickland, J.D.H. and Parsons, T.R. 1972. A Practical Hand Book of Seawater Analysis. Fisheries Research Board of Canada Bulletin 157, 2nd Edition, 310 p.
Teixeira, H., Neto, J.M., Patrício, J., Veríssimo, H., Pinto, R., Salas, F., Marques, J.C., 2009. Quality assessment of benthic macroinvertebrates under the scope of WFD using BAT, the Benthic Assessment Tool. Mar. Pollut. Bull. 58, 1477–1486.
Vieira, L.R., Gravato, C., Soares, A.M.V.M., Mordago, F., Guilhermino, L., 2009. Acute effects of copper and mercury on the estuarine fish Pomatoschistus microps: Linking biomarkers to behaviour. Chemosphere 76, 1416–1427.
Vieira, L.R., Morgado, F., Nogueira, A.J.A., Soares, A.M.V.M., Guilhermino, L., 2018. Integrated multivariate approach of ecological and ecotoxicological parameters in coastal environmental monitoring studies. Ecol. Indic. 95, 1128–1142.
Voulvoulis, N., Arpon, K.D., Giakoumis, T., 2017. The EU Water Framework Directive: From great expectations to problems with implementation. Sci. Total Environ. 575, 358–366.
Wernersson, A.-S., Carere, M., Maggi, C., Tusil, P., Soldan, P., James, A., Sanchez, W., Dulio, V., Broeg, K., Reifferscheid, G., Buchinger, S., Maas, H., Van Der Grinten, E., O'Toole, S., Ausili, A., Manfra, L., Marziali, L., Polesello, S., Lacchetti, I., Mancini, L., Lilja, K., Linderoth, M., Lundeberg, T., Fjallborg, B., Porsbring, T., Larsson, D., Bengtsson-Palme, J., Forlin, L., Kienle, C., Kunz, P., Vermeirssen, E., Werner, I., Robinson, C.D., Lyons, B., Katsiadaki, I., Whalley, C., den Haan, K., Messiaen, M., Clayton, H., Lettieri, T., Carvalho, R.N., Gawlik, B.M., Hollert, H., Di Paolo, C., Brack, W., Kammann, U., Kase, R., 2015. The European technical report on aquatic effect-based monitoring tools under the water framework directive. Environ. Sci. Eur. 27, 7.