Suitability of enzymatic markers to assess the environmental condition of natural populations of *Gambusia affinis* and *Daphnia magna*—a case study

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Abstract In recent years, the use of biochemical markers, especially in the assessment of toxic effects and modes of action, under controlled laboratory conditions has increased. However, transposing their use to in situ monitoring or risk assessment evaluations has encountered barriers, mainly related to the difficulty in interpreting the meaning of biochemical variation. In this work, we aimed at understanding if biochemical marker activities (cholinesterase, glutathione S-transferase and lactate dehydrogenase) can be used to monitor the health status of natural populations of fish (*Gambusia affinis*) and daphnids (*Daphnia magna*). For that, two ponds with different water properties were chosen as study sites, and organisms collected at four sampling periods along the year. The pattern of biochemical marker responses was not the same in the two species, showing higher integrated biochemical marker response values in the winter for *G. affinis* and in the autumn for *D. magna*, suggesting specificities that must be taken into account in biomonitoring programmes by including representative species of several trophic levels. In the case of *G. affinis*, the differences in key physicochemical parameters between the two ponds (especially dissolved oxygen levels) did not seem to affect biochemical marker levels as if organisms were already perfectly adapted to their environment. In general, seasonal variation of water quality seems to have an important role on biochemical marker responses. Several parameters above Environmental Quality Standards were identified such as dissolved oxygen (DO), ammonia, nitrites, sulphides and metals, but eventual responses to these stressors could not be discriminated from natural variation except for particular cases.

Keywords Environmental parameters · Chemical stress · Biochemical markers · *Daphnia magna* · *Gambusia affinis*

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Introduction

In recent years, the use of biochemical markers in the assessment of toxic effects and modes of action,

under controlled laboratory conditions has increased. A biochemical marker, in a perspective of environmental risk assessment, is any biological parameter (a biochemical, physiological, genetic, immunologic or histological measure) that responds to the exposure to a toxicant indicating a deviation of the normal status of the organism, most of the times undetectable at the individual level (Chambers et al. 2002; Morgan et al. 1999). This concept, adopted from medical toxicology, is based on the assumption that low concentrations of a toxicant will have an effect at sub-individual level before observable effects at higher levels of biological organization and thus providing an early sign of disturbance in the ecosystem.

Biochemical markers can be generically classified in three categories (WHO 2001): markers of exposure corresponding to the result of an interaction between a contaminant and a target molecule or cell that is measured; markers of effects, corresponding to an alteration in an organism that can be associated with a possible health condition or disease and markers of susceptibility, indicating that the health of the system is especially sensitive to the challenge of exposure to a xenobiotic compound (NRC 1987).

Biochemical markers have been successfully used in the assessment of pesticides, metals and PAH effects, and several organisms have been used as models, such as insects, crustacea, mollusks, fishes and amphibians (e.g. (Falfushinska et al. 2008; Sanchez and Porcher 2009; Coelho et al. 2011)). The use of biochemical markers in in situ monitoring or risk assessment evaluations has, however, encountered barriers, mainly related to the difficulty in interpreting the meaning of the biochemical variation. Biochemical markers are "early warnings" as they are the first to respond to environmental changes; furthermore, they are highly sensitive comparatively to lethal endpoints and are able to integrate all factors that influence, at a given moment, the physiology of the organism, including effects of available contaminants, abiotic factors and the several interactions that may occur (Barata et al. 2007). This net of factors could never be qualified by chemical analysis; however, it is also a confounding factor when analysing and interpreting the biochemical marker responses. Abiotic parameters, such as temperature (T), dissolved oxygen (DO) concentration or pH, may affect biochemical markers at a

level that can be considered normal or, on the contrary, work as stressors affecting activities in such a way that compromises organisms' fitness or increases their susceptibility to stress (Adams 2000; Vidal et al. 2002; Cailleaud et al. 2007; Charron et al. 2013). The threshold, in terms of biochemical variation, between a fitness impairment or a natural variation is difficult to establish and constitutes a shortcoming for the unambiguous use of biochemical markers as indicators of the biological effects of contamination in field situations. The use of a set of biochemical markers has been the procedure adopted by researchers to overcome this problem and detect in situ effects of toxicants, because an integrated analysis can lead to more robust conclusions. Cholinesterases (EC 3.1.1.8, ChE) are among the most used biochemical markers in lab and field assessments (Olsen et al. 2001) and are especially used to detect contamination by neurotoxic compounds such as organophosphorus and carbamate pesticides. Glutathione S-transferase (EC 2.5.1.18, GST) is a family of enzymes with a key role in the general biotransformation of xenobiotic and endogenous substances (Hyne and Maher 2003). It catalyses the conjugation of reduced glutathione with compounds having reactive electrophilic groups, generating less toxic and more hydrophilic molecules and is induced by compounds such as organochloride pesticides, PAHs and PCBs (Cailleaud et al. 2007). Lactate dehydrogenase (EC 1.1.1.27, LDH) is involved in the carbohydrate metabolism, catalysing the oxidation of lactate into pyruvate (Diamantino et al. 2001).

This work aimed at understanding if enzymatic biochemical marker activities can be used to monitor the health status of natural populations of fish (Gambusia affinis) and daphnids (Daphnia magna). For that, two ponds with different water properties were chosen as case study, and organisms were collected at four sampling periods (representative of the four seasons) along the year. To attain this major goal, three specific objectives were delineated: (i) to assess biochemical marker variation among ponds and among seasons; (ii) to identify water physicochemical parameters acting as stress factors (based on thresholds already established in the legislation) and verify if biochemical markers respond specifically to these stressors and finally (iii) to verify if the pattern of response varies between

organisms belonging to different trophic levels (fish and crustaceans).

Materials and methods

Sampling sites

Two ponds located at the campus of the University of Aveiro, Portugal, were selected as study sites. The natural pond (NP) is a natural water body of approximately 3955 m^2 receiving pluvial waters. The artificial pond (AP) is a concrete tank with 5317 m^2 whose waters are more prone to stagnation depending essentially on rain as water supply. Both ponds have the features of a lentic ecosystem with the establishment of organisms belonging to different trophic levels (Fig. 1).

The procedures described in the present paper respect national and international safety regulations and ethical principles for animal welfare.

Field procedure

Sampling

Adult individuals of the cladoceran species *D. magna* and of the fish species *G. affinis* were collected in both ponds using a zooplankton and a hand net, respectively. Sampling comprised four episodes: summer (August 2008), autumn (November 2008), winter (February 2009) and spring (May 2009). In the laboratory, individual fish and pools of 15 daphnids were snap frozen in liquid nitrogen in microtubes and stored at −80 °C until biochemical marker analysis.

Determination of physicochemical parameters

At each sampling episode, water samples were collected for physicochemical characterization in the laboratory. In the impossibility of immediate analysis, samples were frozen at −80 °C. Analyses were performed, using the Test Kit models from Hach, and
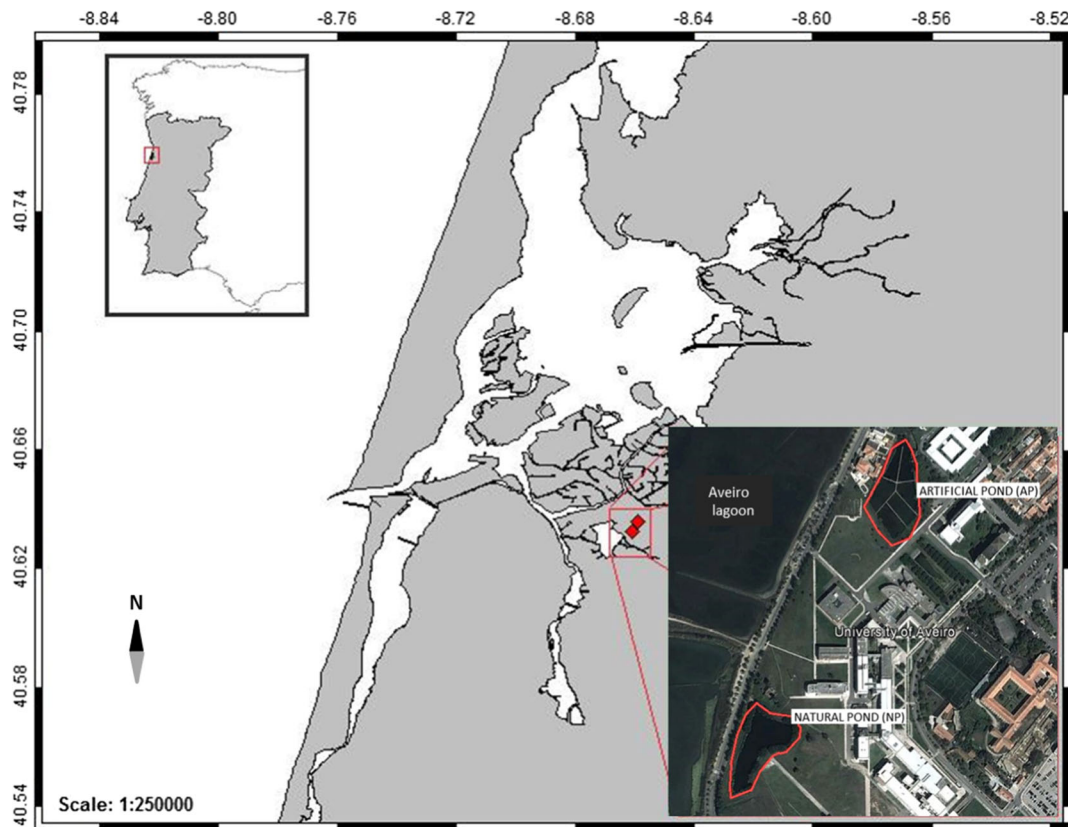
Fig. 1 Location of the study sites, in Aveiro, Portugal. Natural pond: 40° 37′ 57.4″ N 8° 39′ 40.2″ W; artificial pond: 40° 38′ 07.9″ N 8° 39′ 33.4″ W

the following parameters were determined: total hardness (method 8226), alkalinity (method 8221), turbidity (method 8237), sulphides (method 8131), sulphates (method 8051), reactive phosphor (method 8048), nitrites (method 8507), nitrates (method 8171) and ammonia (method 8038). Each parameter was determined according to the methods described on the Hach DR/2000 Spectrophotometer Handbook, and the measurements were made using a Hach DR/2000 Direct Reading Spectrophotometer.

Conductivity, pH, DO and T were measured in the field at each sampling episode using portable meters (LF 330, pH 330, OXI 330 and sets from WTW, Weilheim, Germany, respectively).

Element content of the water

For element analysis, water samples were acidified (pH <2) with nitric acid prior being frozen. The following elements were analysed by inductively coupled plasma mass spectrometry (ICP-MS) at the certified Central Laboratory of Analysis from the University of Aveiro (accreditation no. L0627-1): aluminium (Al), chromium (Cr), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), mercury (Hg), cadmium (Cd) and lead (Pb).

Daphnia magna laboratory culture

As *D. magna* was only present at the AP, a lab culture was taken as the control population. *D. magna* has been reared in the Laboratory of Ecotoxicology at Department of Biology, University of Aveiro for more than 10 years in American Society for Testing and Materials (ASTM) hard water (ASTM 1980) enriched with an organic additive. The photoperiod is 16 h light/ 8 h dark and the T 20 ± 1 °C. The culture media has a total hardness of 175.41 ± 5.53 mg/L $CaCO_3$, pH of 8.15 ± 0.27 and a conductivity of 577.63 ± 9.01 μS/cm. Medium was renewed three times per week, and daphnids were fed daily with *Chlorella vulgaris* at a concentration of 3.0×10^5 cells/mL. Sampling for biochemical marker analysis was done by pooling 15 individuals (21 days old) originated from a third brood in microtubes, which were then snap frozen in liquid nitrogen and stored at −80 °C until biochemical marker analysis.

Biochemical marker analyses

Post-mitochondrial supernatant

On the day of enzymatic analysis, samples were defrosted on ice and adequate buffer solution added: Phosphate buffer (0.1 M, pH=7.4) was used for ChE, GST and CAT analysis, and Tris/NaCL buffer (0.1 M; pH=7.2) was used for LDH analysis. Amount of added buffer was 1.5 mL for fish replicates and 0.5 mL for daphnid replicates. A minimum of eight replicates was used per site. Each replicate was homogenized (using a Ystral homogenizer) being kept in ice during the process and centrifuged at $10,000\times g$ for 20 min (4 °C) in the case of ChE or GST analyses and at $1700\times g$, 3 min (4 °C) in the case of LDH analyses. All the enzymatic assays were performed on the supernatant obtained.

Protein concentration

Protein concentration in the replicates was determined in quadruplicate by the Bradford method (Bradford 1976), at 595 nm, using γ-globulin as standard and then adjusted to 0.5 mg/L of protein using the respective buffer to dilute. Final protein concentration was determined again for confirmation.

Enzymatic analysis

Total ChE activity was determined at 414 nm according to the method of Ellman (Ellman et al. 1961), adapted to microplate (Guilhermino et al. 1996), using 0.05 mL of homogenate and 0.250 mL of the reaction solution (1 mL of 10 mM 5.50-dithiobis-2-nitrobenzoic acid solution with sodium hydrogen carbonate, 0.2 mL of 0.075 M acetylcholine solution and 30 mL of 0.1 M phosphate buffer). Different ChE isoforms may be present in organisms. In this work, they will be generically referred as ChE for both species studied although previous work showed that in the case of *Gambusia*, the predominant form is acetylcholinesterase (Nunes et al. 2005) while in *D. magna*, both acetylcholinesterases and pseudocholinesterases seem to co-exist (Diamantino et al. 2003).

GST activity was determined at 340 nm by the method of Habig et al. (Habig et al. 1974), adapted to microplate (Frasco and Guilhermino 2002), using 0.1 mL of homogenate and 0.2 mL of the reaction mixture (10 mM

reduced glutathione and 60 mM 1-chloro-2.4-dinitrobenzene).

LDH activity was determined at 340 nm by the method of Vassault (Vassault 1983) adapted to microplate by Diamantino et al. (Diamantino et al. 2001), using 40 µL of homogenate, 250 µL of NADH (0.24 mM) and 40 µL of pyruvate (10 mM).

Enzymatic activities were determined in quadruplicate and expressed as nanomoles of substrate hydrolysed per minute per milligram of protein. A Labsystem Multiskan EX microplate reader was used for all biochemical determinations.

Data processing

Statistical analysis

Sigma Stat 3.1 statistical package was used for statistical analyses (SPSS 2004). In the case of *G. affinis*, differences in biochemical marker activities between ponds (natural vs artificial) and seasons were evaluated using a two-way ANOVA followed by the Tukey test. In the case of *D. magna*, differences in biochemical marker activities between seasons were evaluated through one-way ANOVA followed by the Tukey test. Transformation of data was done in the cases the Kolmogorov-Smirnov normality test failed.

A redundancy analysis (RDA) was performed on endpoint datasets (as species data) including physicochemical water parameters (as environmental data) following the log-transformation of data. Multivariate analysis was performed using CANOCO 4.5 (Ter Braak and Smilauer 2002).

Calculation of the integrated biochemical marker response

The integrated biochemical marker response is an index which combines the responses of all biochemical markers measured in the study. The calculation involves a first step of data standardization which allows the direct comparison of different biochemical marker responses. Star plots are then used to represent the obtained scores (standardized data) of a given biomarker at different sampling episodes or the scores of all biochemical marker responses for a given sampling episode. In this later case, the area of the star plot is the integrated biochemical marker response (IBR). The steps involved in the calculation of scores and IBRs were performed as described in Beliaeff and Burgeot (2002). IBR values were then divided by the number of biomarkers used (3) and consequently expressed as IBR/n, so that it can be used as a general biomarker index (as described in Broeg and Lehtonen (2006)). For the calculations of the scores, it has to be defined if a given biomarker responds to a situation of stress by increasing or decreasing its activity. Thus, in this work, it was assumed that an inhibition would occur in the case of ChE, and on the contrary, GST and LDH would respond to stress by increasing their activities.

Results

Physicochemical parameters and elements' content in the two ponds

The artificial pond was characterized (especially in warm seasons) by higher values of conductivity, ammonia, alkalinity, nitrates, nitrites, reactive phosphor, sulphates and also the metals Mn and Fe while consistently presented lower values of pH, DO and Zn than the natural pond. From these parameters, DO, ammonia and nitrites are consistently above (or below in the case of DO) the Environmental Quality Standards (EQS) established in the literature (EPA 1999; European Commission 2000; European Union 2006, 2008; UKTAG 2008), meaning that these factors can potentially behave as stress factors for aquatic life and that the ecological fitness of the ecosystem can be threatened in this lake (Table 1, highlighted values).

Regarding seasonality and for both ponds, parameters such as pH, T, ammonia and reactive phosphor reached higher values on summer and spring, while sulphides, nitrates and Al reached higher values on winter and autumn. Sulphides and Al are of special concern since they were above EQS values (Table 1). Metals such as Cr, Ni, Cu, Zi, and Pb showed an increase in autumn; however, Cu, Zn and Pb were above EQS levels almost all the four seasons.

Gambusia affinis biochemical marker responses

Biochemical marker activities measured in *G. affinis* seemed to depend more on the season than on the pond except for some cases further to discuss (Fig. 2). The two-way ANOVA performed (Table 2) indicated differences between seasons for all biochemical markers

Table 1 Water physicochemical parameters measured in the several sampling episodes (*NP* natural pond, *AP* artificial pond, *ESV* environmental screening value)

Unit	Season	Summer		Autumn		Winter		Spring		EQS
	Pond	NP	AP	NP	AP	NP	AP	NP	AP	
	physico-chemical parameters									
	pH	9.08	7.65	8.02	7.59	8.13	7.47	8.69	7.54	6-9 [26]
ºC	**Temperature**	20.3	19.4	14.5	14.1	12.4	12.4	19.9	21.1	
µS/cm	**Conductivity**	295	660	351	729	393	564	354	679	
mg/L	**DO**	9.8	1.8	10.4	2.1	9.1	6.3	4.95	1.15	7 [26]
mg/L NH3 Ness	**Ammonia**	0.13	12.3	0.04	3.55	0.08	8.3	0.15	15.6	1.270 [24]
mg/L CaCO3	**Alkalinity**	137	205	88.2	155	116	201	87.0	193	
mg/L N NO3- M	**Nitrates**	0.0	0.4	0.8	1.5	0.6	2.2	0.3	0.4	50 [25]
mg/L N NO2- L	**Nitrites**	0.006	0.027	0.013	0.066	0.013	0.09	0.036	0.107	0.06 [24]; 0.03 [26]
mg/L PO43- PV	**Reactive Phosphor**	0.25	17.2	0.20	4.40	0.33	4.00	0.29	14.5	
mg/L SO42-	**Sulfates**	19	32	23	38	22	29	23	31	1000 [47]
mg/L S2	**Sulfides**	< 0.00	0	0.002	0.006	0.004	0.007	< 0.00	< 0.00	0.002 [24]
FTU	**Turbidity**	1.0	3.0	0.0	0.0	0.0	18	1.0	12	
mg/L CaCO3	**Total Hardness**	14	16	8	15	13	11	14	10	
	elements									
Ppb	**Aluminium**	60	47	114	52	66	91	41	77	87 [24]
Ppb	**Chromium**	1.1	1.5	1.7	1.9	0.99	1.4	0.88	1.8	5-50 [27]
Ppb	**Manganese**	4.3	55	4.9	18	6.7	78	19	66	80 [24]
Ppb	**Iron**	35.0	227	77.0	390	53.0	489	117	478	1000 [27]
Ppb	**Cobalt**	0.36	0.46	0.23	0.32	0.23	0.36	0.17	0.42	3 [24]
Ppb	**Nickel**	1.3	1.8	1.6	2.1	0.8	1.4	0.76	2	87.71 [24]
Ppb	**Copper**	2.7	0.78	4.8	60	2.6	2	25	5.4	1 [27a]
Ppb	**Zinc**	30	7.7	67	37	26	15	40	21	8 [27a]
Ppb	**Arsenic**	3.7	2.1	2.1	3	0.8	1.7	1	1.9	50 [27]
Ppb	**Cadmium**	<0.05	<0.05	0.05	0.25	<0.05	<0.05	<0.05	0.052	0.45 [28a]
Ppb	**Mercury**	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	0.07 [28]
Ppb	**Lead**	1.5	1.6	2.4	8.1	0.8	1.3	6	2.3	1.32 [24]

Values above the Environmental Quality Standard (EQS) (or below in the case of dissolved oxygen (DO)) are highlighted in *grey*. EQS values are followed by the citation number

[a] Corrected by water hardness

tested while differences between ponds were observed for ChE and LDH only. Results from the RDA are summarized in Fig. 3, showing a strong correlation between biomarker levels and water properties on the first two axes, which explained 99.8 % of the total variability (98.7 and 1 %, respectively).

Cholinesterase activity (Fig. 2a) was higher in summer at the NP compared to any other sample. Within the AP, ChE presented lower values in winter and spring which in the RDA analysis seemed to be associated with the high values of nitrites, ammonia, Mn and Fe and low levels of DO (Fig. 3, Table 1).

No differences between GST activities were observed between ponds (Fig. 2b). For both ponds, activities were lower in summer, increasing towards winter and spring in a pattern strongly associated with Fe and nitrites values.

Lactate dehydrogenase activities (Fig. 2c) were higher in autumn, winter and spring (only AP), which was positively associated with Al, sulphides and nitrate values and negatively associated with T.

Calculation of scores and IBRs allowed an integrated visualization of the biochemical marker responses (Fig. 4). The minimal biological response was observed
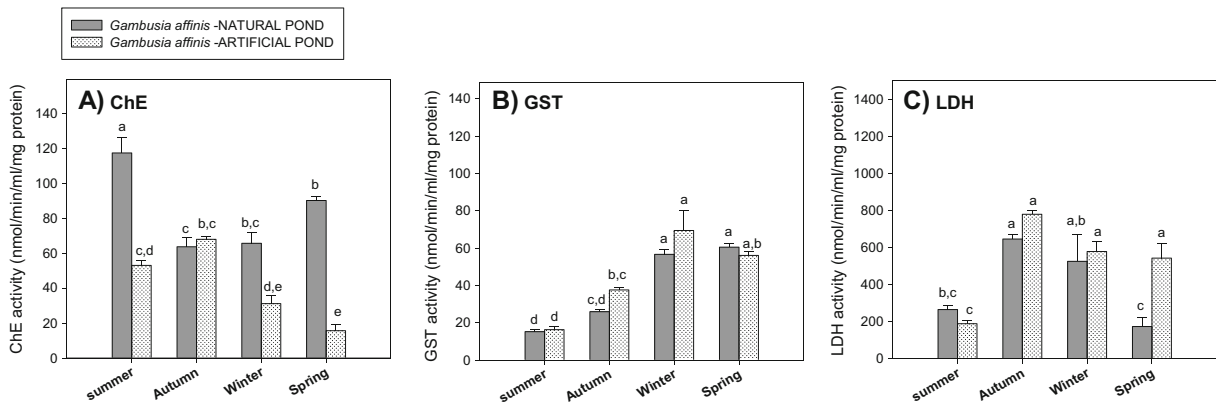
Fig. 2 Biomarker activities of *Gambuzia affinis* (mean±standard error) measured after each sampling episode. *Different letters above the bars* mean significant differences between groups (Tukey test, $p<0.05$)

consistently for all biochemical markers in summer and the maximal in the winter. This pattern of response was very similar between lakes, being however more marked in the AP. The IBR star plot confirms the same pattern (Fig. 4d).

Daphnia magna responses

No daphnids were found in the NP; thus, results from the AP were compared with lab values as a reference control (Fig. 5). The one-way ANOVA performed (Table 2) indicated differences between seasons for all biochemical markers tested. The RDA (Fig. 6) could only be performed with data from summer, autumn and spring (due to lack of data for GST in the winter). A strong correlation between biomarker levels and water properties on the first two axes was observed, explaining 100 % of the variability (81.6 and 18.4 %, respectively).

Cholinesterase activities (Fig. 5a) of daphnids collected in the summer, autumn and spring were lower than the ones from the lab control. In summer and spring, this seemed to be associated with the low levels of DO and high levels of conductivity, T, reactive phosphorous and ammonia while in autumn with the high levels of Cu, Zn and Pb which are above EQS (Table 1). The high concentrations of the metals Cu, Zn, Cd and Pb and As was also associated with the increased activity of GST in the autumn compared to values from lab culture (Fig. 5b and Fig. 6); moreover, depression of GST activities in the summer and spring seems to be associated with higher temperatures, reactive phosphorous and ammonia contents. LDH activity was higher in the lab control and in the winter compared to the other seasons (Fig. 5c).

Due to missing data for GST in winter, star plots could only be done for lab control, spring, summer and autumn (Fig. 7). The maximal biological response was observed in summer and autumn for AChE

Table 2 Analysis of variance of biomarker data for *Gambuzia affinis* and *Daphnia magna* from the natural and artificial ponds, during the sampling episodes (seasons)

Factors	Two-way ANOVA of biomarker activities measured in *G. affinis*		
	ChE	GST	LDH
Season	$F_{3,\,64}=20.8$; $p<0.001$	$F_{3,\,63}=57.4$; $p<0.001$	$F_{3,\,54}=31.8$; $p<0.001$
Pond	$F_{1,\,64}=125.5$; $p<0.001$	$F_{1,\,63}=3.2$; $p=0.079$	$F_{1,\,54}=9.01$; $p=0.004$
Season × pond	$F_{3,\,64}=22.2$; $p<0.001$	$F_{3,\,63}=2.00$; $p=0.126$	$F_{3,\,54}=5.96$; $p=0.002$
Factor	One-way ANOVA of biomarker activities measured in *D. magna*		
	ChE (ln che)	GST	LDH (sqrt LDH)
Season	$F_{4,\,35}=22.1$; $p<0.001$	$F_{3,\,28}=34.0$; $p<0.001$	$F_{4,\,36}=378$; $p<0.001$

ChE cholinesterase, *GST* glutathione S-transferase, *LDH* lactate dehydrogenase

Fig. 3 Species-environment triplot diagram from the redundancy analysis. Endpoint datasets of *Gambuzia affinis* as species data (ChE, LDH and GST) and physicochemical water parameters as environmental variables: turbidity, temperature, total hardness, alkalinity, conductivity, reactive phosphor, ammonia, nitrates, sulphides, nitrites, dissolved oxygen (DO concentration), pH, manganese, copper, lead, zinc, iron and aluminium
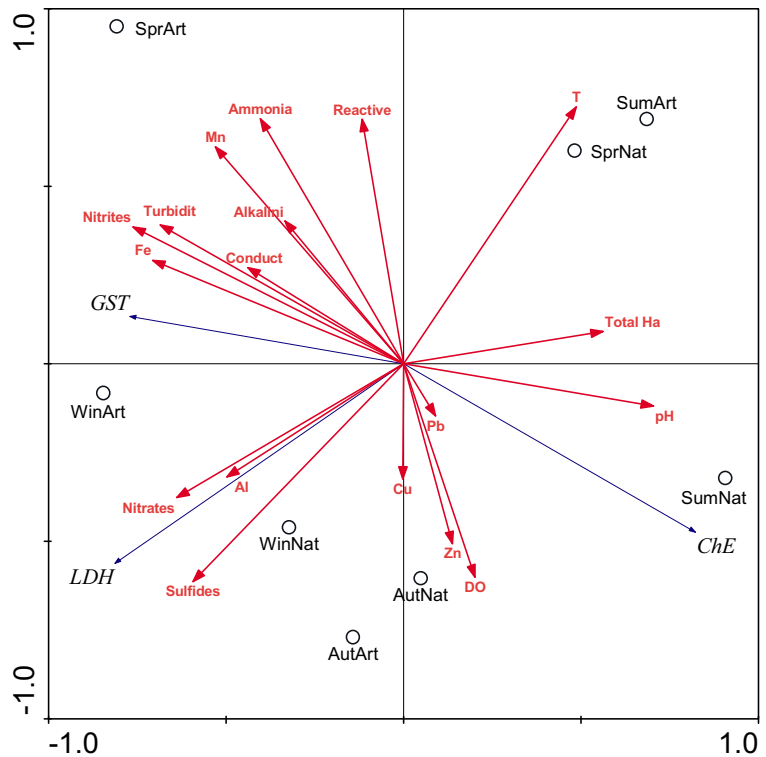
(Fig. 7a), in autumn for GST (Fig. 7b) and in the lab samples for LDH (Fig. 7c). Thus, each biomarker had a specific pattern of response. IBR (Fig. 7d) showed that the response, when integrating all biochemical markers analysed, was more intense in autumn.

Discussion

Gambusia affinis

The biochemical marker activities measured in *G. affinis* were within the range of basal levels measured in *Gambusia holbrooki* collected from reference sites in

the study of Castro et al. (Castro et al. 2004), of approximately 80, 40 and 300 nmol/min/mg protein for ChE, GST and LDH, respectively (values taken from graphs).

Biochemical markers measured in the two populations of *G. affinis* showed to have a higher biological response in winter as indicated by the integrated biomarker response (Fig. 4d), especially in the AP samples. However, this response did not seem to be associated to any particular chemical component present at that time of the year but rather explained by natural seasonal enzymatic variation. Even elements that are above EQS such as sulphides, nitrites or some metallic elements did not seem to sufficiently justify this winter response. Natural seasonal variation of enzymatic
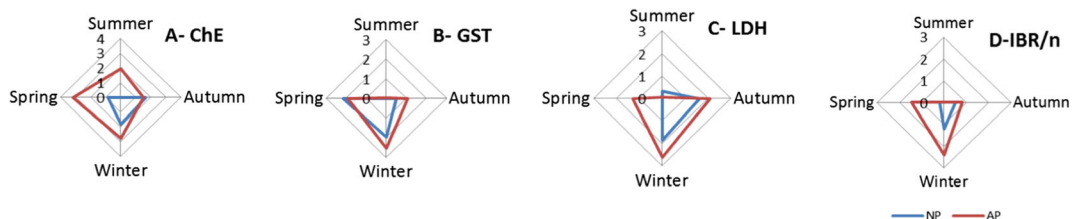
Fig. 4 Cholinesterase (ChE) (**a**), glutathione S-transferase (GST) (**b**) and lactate dehydrogenase (LDH) (**c**) and integrated biomarker response divided by the number of biomarkers tested (3) (IBR/n)

(**d**) star plots for *Gambuzia affinis* collected from the natural pond (NP) and artificial pond (AP) in summer, autumn, winter and spring
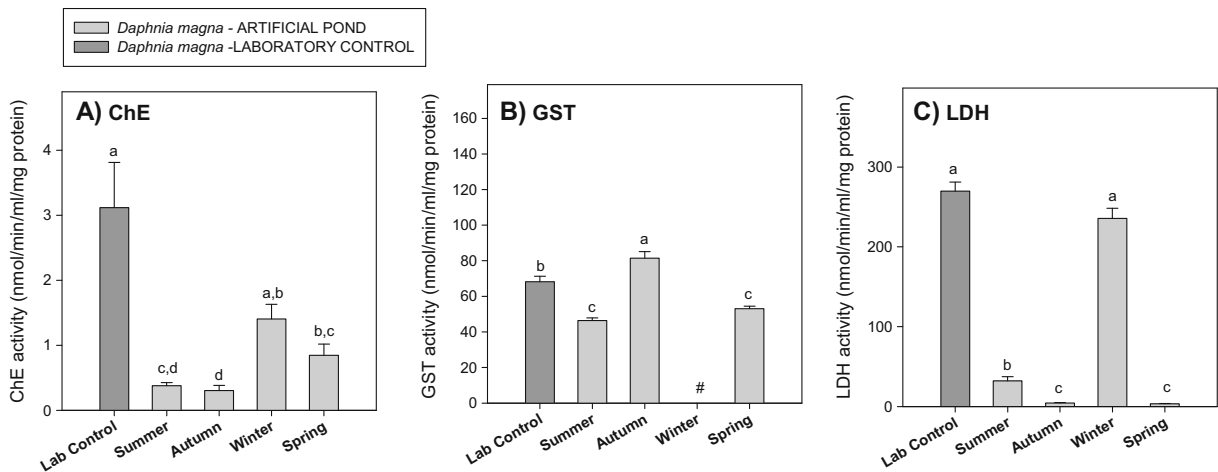
Fig. 5 Cholinesterase (ChE) (**a**), glutathione S-transferase (GST) (**b**) and lactate dehydrogenase (LDH) (**c**) activities in *Daphnia magna* (mean±standard error) measured at each sampling episode in the summer, autumn, winter and spring and from a laboratory culture. *Different letters above the bars* mean significant differences between groups (Tukey test. $p<0.05$). "*Number sign*" indicates information not available
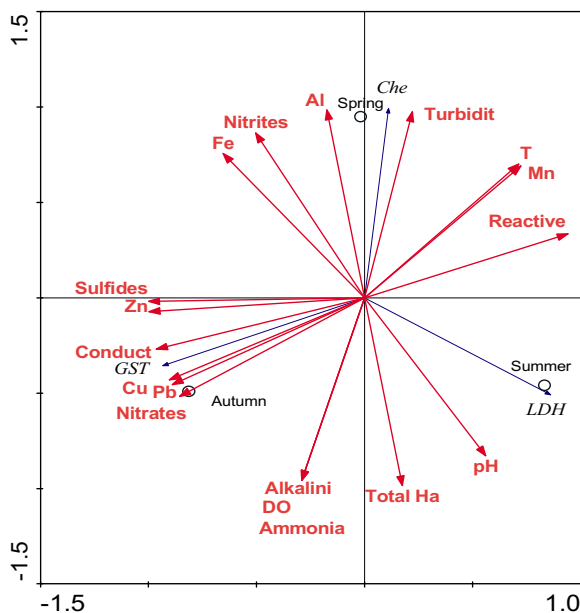
activity or environmental influence has been already mentioned in several studies (Barhoumi et al. 2012; Palais et al. 2012). For instance, LDH and GST activities of *Cyprinus carpio* collected in the Meriç Delta (Turkey) also showed seasonal variation (Gungordu

Fig. 6 Species-environment triplot diagram from the redundancy analysis. Endpoint datasets of *Daphnia magna* as species data (ChE, LDH and GST) and physicochemical water parameters as environmental variables: turbidity, temperature, total hardness, alkalinity, conductivity, reactive phosphor, ammonia, nitrates, sulphides, nitrites, dissolved oxygen (DO concentration), pH, manganese, copper, lead, zinc, iron and aluminium

et al. 2012). Parameters such as food availability, diet composition, DO, T and pH seem to affect oxidative stress enzymes (Sanchez et al. 2008; Gungordu et al. 2012) while age and reproductive status are known to affect ChE in fish (Phillips et al. 2002).

Thus, this general variation in the biochemical markers analysed is more prone to reflect seasonal environmental variation or physiological condition of the organisms, and a link to the fitness of the organisms is, thus, difficult to be established.

A more clear difference between ponds was expected to be detected, since at a first glance, the AP seems more disturbed than the NP as it presents higher values of ammonia, nitrates, nitrites, reactive phosphor, sulphates and also the metals Mn, Fe, Co and Ni while consistently presents lower values of DO. However, only ammonia, nitrites and DO exceed (or are above in the case of DO) the EQSs. The content in these elements probably explained the slightly higher IBR found at the AP, but it is slightly detected when analysing biochemical marker responses individually.

Biochemical marker responses analysed individually may however reveal some clues regarding particular elements acting as stress factors. For instance, ChE depression during winter and spring at the AP is associated to high levels of nitrites, ammonia, Mn and Fe. Nitrites and ammonia are in these cases above EQS values. There are evidences that nutrient enrichment may cause inhibition of ChE such as in the study of Yadav et al. (Yadav et al. 2009) where the fish *Channa*
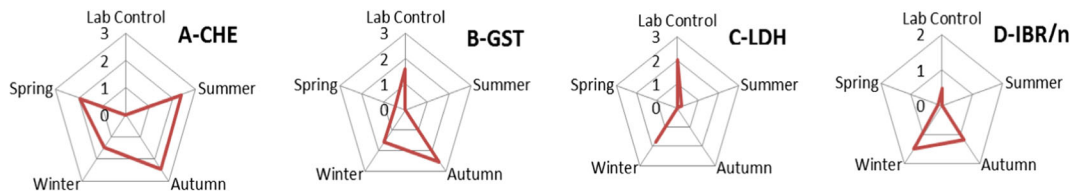
Fig. 7 Cholinesterase (**a**), glutathione S-transferase (GST) (**b**) and lactate dehydrogenase (LDH) (**c**) and integrated biomarker response divided by the number of biomarkers tested (3) (IBR/n) (**d**) star plots for *Daphnia magna* collected from the natural pond (NP) and artificial pond (AP) in summer, autumn, winter and spring

striatus was exposed to fertilizer industry effluents. ChE inhibition associated with high levels of Mn and Fe is also expected as this enzyme is known to respond to metal exposure in general (Frasco et al. 2005) and Mn (Falfushynska et al. 2011) in particular. Other parameters with whom ChE seemed to be associated in the RDA graph, such as pH and DO, did not seem to influence it according to Sanchez et al. (Sanchez et al. 2008).

GST activity did not differ between ponds presenting a smooth variation across seasons which, in the RDA graph, showed to be associated with levels of nitrites and Fe. Although such association is possible (it is known that both metals (Castro et al. 2004) and nutrients (Lima et al. 2007) may interfere with phase I biotransformation processes in fish), it is still difficult to prove and distinguish from natural variation.

LDH activities were higher in autumn and winter when compared to other seasons which may be a general stress response to high levels of Al, sulphides and nitrates or, a direct influence of temperature, agreeing with the work of Gungordu et al. (Gungordu et al. 2012) in which the LDH of *C. carpio* collected in the Meriç Delta (Turkey) presented higher activities in cold seasons. Moreover, the physiological status of the organism is also known to influence LDH activity along the year. For instance in the yellow perch (*Perca flavescens*), LDH activity is lower during spawning activity (Schoenebeck and Brown 2012).

Daphnia magna

The biochemical marker activities measured in *D. magna* were within the range of basal levels described in the literature, with approximately 0.45–9 nmol/min/mg protein for ChE and 67.3–350 nmol/min/mg protein for GST (levels strongly vary with the age of the daphnids) according to Jemec et al. (Jemec et al. 2010). In addition, the LDH basal level reported by Diamantino et al. (Diamantino et al. 2001) was also

within the range with approximately 250 nmol/min/mg protein (value from graph).

The integration of results from different biochemical markers (Fig. 7d) showed a higher response in winter and autumn. However, the pattern of response among biochemical markers was different. In the case of ChE, the activity measured from daphnids from the AP was much lower than in those from the lab control and among seasons was lower in summer and autumn. However, associations with DO, conductivity and hardness verified in the RDA analysis are not likely to explain these differences (no clear evidences were found in literature supporting the influence of these factors on ChE). Thus, differences are more likely to be due to seasonal variation (which occurs for many invertebrates according to Berra et al. (Berra et al. 2004)) and due to possible different ages of organisms (Domingues et al. 2010).

GST increased activity in autumn probably reflected the higher concentrations of metals (Cu, Ar, Zn, Cd and Pb) in the AP at this time of the year (Jemec et al. 2008). Moreover, the depression of activities in the summer and spring seemed to be associated with higher temperatures, reactive phosphorous and ammonia contents. According to Domingues et al. (Domingues et al. 2010), GST in invertebrate organisms is influenced by environmental factors in a not very predictable way, and thus, direct links between enzymatic responses and measured parameters are difficult to be established.

Although an increment in LDH activity was expected as a response to stress, in our work, LDH of daphnids collected in summer, autumn and spring was much lower than the activity measured in the control population. According to Diamantino et al. (Diamantino et al. 2001), there is a negative correlation between LDH activity and environmental DO that can explain this decrease. In our work, such a correlation was observed, although it cannot be verified in the RDA graph since winter was not taken into account in that analysis. Moreover, many examples in the literature show that LDH in many cases responds to environmental stress by

inhibiting and not inducing its activity (Coelho et al. 2011; Barbosa et al. 2013).

For the AP, where a comparison of responses between species can be done, it can be observed that biochemical markers were more responsive in winter for *G. affinis* and in autumn for *D. magna.* This suggests that biomonitoring programmes should contemplate analyses of species belonging to different trophic levels as different patterns of responses among species may indicate different sensitivities and different risks associated with the respective populations.

In this work, several stress factors were identified in both ponds, including low levels of DO and ammonia (only in the AP) and nitrites, sulphides and metal elements in both ponds. A clear response to these stress factors was not possible to obtain by measuring ChE, GST or LDH levels in *G. affinis* and *D. magna* collected at the two sites. This happens because biochemical markers, although able to respond to several types of stress factors, are also related to natural environmental temporal and spatial variations being modulated by temperature, food availability (Cailleaud et al. 2007) and physiological status of the organisms such as age and reproductive status. This complex net of factors modulates biochemical marker basal levels, confounding the response to the identified stress factors. This inability to distinguish biochemical marker responses to chemicals from responses to natural variability may compromise their use in biomonitorization approaches (Kopecka and Pempkowiak 2008).

Besides natural variability, other factors not contemplated in this study may be interfering with enzymatic responses as it is the case of interactive effects between stress factors (chemical or environmental) that may alter the expected toxicity (Ferreira et al. 2008; Ferreira et al. 2010) and also the existence of other types of pollutants such as pesticides.

Besides the response to the parameters identified as stress factors (above EQS), a more clear response between ponds was expected since they have very different water physicochemical properties especially in what concerns DO, ammonia, nitrites, reactive phosphorous and sulphates. Such differences were not reflected in the activities of biomarkers measured suggesting that organisms are already well adapted to those environmental conditions. To test the hypothesis that local organisms are physiologically adapted to the components (chemical and environmental) identified, further work should include the deployment of laboratory organisms in both sites or perform cross-transplant experiments to assess the sensibilities at the two sites.

The IBR index was a useful tool especially in the case of *G. affinis* where a difference between ponds could be detected which was not very obvious by analysing biochemical markers individually. However, the main shortcoming of this index is that the expected biomarker behaviour has to be predefined (induction or inhibition) while many times, particularly for LDH and GST, responses to pollutants may be an inhibition, an induction or both (bell-shape curve).

Conclusion

In this work, the levels of ChE, GST and LDH of natural populations of *G. affinis* and *D. magna* were monitored along the year in two ponds with different physicochemical water properties. The pattern of biochemical marker responses was not the same in the two species with higher IBR values in the winter for *G. affinis* and in the autumn for *D. magna*, suggesting specificities that must be taken into account in biomonitorization processes, by including species representative of several trophic levels.

In the case of *G. affinis*, the differences verified in key physicochemical parameters between the two ponds (especially DO levels) did not seem to affect biochemical marker levels as if organisms were already perfectly adapted to their environment. On the other hand, seasonal variation of water quality and/or physiological status of the organisms may have an important role on biochemical marker responses. Several parameters above EQS were identified (e.g. DO, ammonia, nitrites, sulphides and metals), but eventual responses to these stressors could not be discriminated from natural variation. However, in particular cases, important associations could be established as the one between inhibition of ChE in winter and spring in *G. affinis* from the AP and high levels of nitrites, ammonia, Mn and Fe; the induction of GST activity in autumn in *D. magna* from the AP and high levels of metals and the decreased LDH activity in summer, autumn and spring in *D. magna* from the AP and low DO levels.

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