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AREA 5 • BIOMONITORING • REVIEW ARTICLE

Biochemical biomarkers in environmental studies—lessons learnt from enzymes catalase, glutathione *S*-transferase and cholinesterase in two crustacean species

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Abstract
Background, aim and scope For reliable environmental risk assessment of pollutants, knowledge on the effects at different levels of biological organisation is needed. During the early days of biomarker research in environmental studies approximately two decades ago, biochemical biomarkers were considered as the most promising tool for such purposes. Among these, three enzymes have often been studied: catalase (CAT), glutathione *S*-transferase (GST) and cholinesterase (ChE). However, despite their intensive research, their measurements in invertebrates have not been commonly applied in environmental risk assessment (ERA) or for regulatory purposes.
Main features In the present review, we summarise our past experiences in biochemical biomarker research in two crustacean species: water flea *Daphnia magna* and terrestrial isopod *Porcellio scaber*. This is to orientate their use and to provide recommendations for the use of novel biomarkers in environmental studies, such as proteomic or genomic responses.
Results and discussion We assessed the intrinsic properties of biochemical biomarkers CAT, GST and ChE in the *D. magna* and the isopod *P. scaber*. It was found that they are not in agreement with the expectations that were previously given for their use in environmental studies. To advance their use in environmental risk assessment, we suggest that based on their properties, their role should be more specifically defined. ERA includes several distinct steps, among them hazard identification, effect assessment and finally risk characterisation, each of which requires a different type of toxicity data. We recommend that the use of biochemical markers is most appropriate for hazard identification because this is a procedure whose purpose is to characterise the potential hazard of the substance in question and is more flexible in terms of using different tools. Furthermore, our results imply that biochemical markers are not always more sensitive than whole-organism responses, as was anticipated. Their sensitivity depends on the mode of action, duration of exposure and test species. Therefore, we suggest that combining both a battery of biomarkers from different levels of biological organisation and an array of biomarkers within a single level could identify hazard adequately.
Conclusions The lesson learnt from biochemical biomarkers in environmental studies utilizing crustacean model species is that, for successful application of each group of biomarkers, their intrinsic properties are needed to be known before an (eco)toxicity study is designed. We suggest that a substantial body of experience obtained with biochemical biomarkers should be exploited to new emerging biomarkers in environmental studies in order to facilitate their application.
Recommendations and perspectives The future of biomarkers lies in a combination of traditional biochemical and new-generation biomarkers. The latter are not only a potential replacement for existing biomarkers but will also provide new knowledge which might encourage renewed research and development of traditional biomarkers. For research purposes, complete ecotoxicity information should include contributions from molecular fingerprint of an

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organism, as well as whole organism, population and ecosystem responses. Still, the type of biomarkers used for routine purposes will depend on their reproducibility, their ease of use, robustness, affordability of the methodology and the type of chemicals, organisms and ecosystem of interest.

Keywords Biochemical biomarker · Catalase · Cholinesterase · *Daphnia magna* · Environmental risk assessment · Glutathione *S*-transferase · Hazard · Marker · Metals · Pesticides · Terrestrial invertebrate *Porcellio scaber* · Toxicogenomics · Toxicoproteomics

1 Background, aim and scope

For reliable hazard identification of environmental contaminants/pollutants, knowledge of the effects on different levels of biological organisation is necessary. As a consequence of their ability to identify causal mechanisms potentially responsible for effects at higher levels of organisation, biochemical biomarkers used to be considered the most promising tools for ecotoxicological applications (Peakall and Walker 1994; Adams 2002). Enzyme activities and other sub-cellular components are most commonly included in this group of biomarkers, and much work has been done during the last two decades to establish and promote their application (Adams 2002). In invertebrates, a lot of attention was given to three enzymes: catalase (CAT), glutathione *S*-transferase (GST) and cholinesterase (ChE; Guilhermino et al. 1996; Livingstone 1998; Barata et al. 2005). The inhibition of ChE by organophosphorus and carbamate pesticides, as well as metals and detergents, results in an over-accumulation of the neurotransmitter acetylcholine, thus causing prolonged electrical activity at nerve endings and ultimately leading to death. The two antioxidant enzymes, GST and CAT, are important in the prevention of oxidative stress damages. GST catalyses the conjugation of glutathione with xenobiotics, including organophosphorus pesticides, and the cytotoxic aldehydes produced during lipid peroxidation (Booth and O'Halloran 2001). Catalase decomposes the hydrogen peroxide. In case the activities of these two enzymes are diminished, the organism could be exposed to very high levels of reactive oxygen species, leading to oxidative damages of principal cellular components, such as lipids, proteins and DNA (Halliwell and Gutteridge 2007).

Currently, ecotoxicogenomics and ecotoxicoproteomics are viewed as the next steps in the evolution of environmental biomarkers, and great expectations are associated with such 'omic' techniques (Bishop et al. 2001; Moore 2002; Neumann and Galvez 2002; Calzolai et al. 2007; Scholz et al. 2008). These novel approaches are based on measurements of gene or protein expression following exposure to a chemical and result in an exposure fingerprint, which provides information concerning the response of cells and organisms to changes in the external environment (Calzolai et al. 2007). Many applications of this approach in environmental studies have been suggested and include investigation of a pollutant's mechanism of action, fast screening of unknown pollutants in the environment (i.e. stressor identification), risk assessment and improved analysis of the effects of mixtures of pollutants (Snell et al. 2003).

Among invertebrates, much attention was given to the use of these novel techniques in aquatic crustacean *Daphnia magna*, and it has already been suggested as a leading model invertebrate in ecotoxicogenomics (Poynton et al. 2007; Heckmann et al. 2008; Shaw et al. 2008). Such recent molecular investigations on *D. magna* include studies on heavy metals (Connon et al. 2008), anti-inflammatory drugs (Heckmann et al. 2008), pentachlorophenol and β-naphthoflavone (Watanabe et al. 2007). These studies focused mainly on providing mechanistic insight into the mode of action of stressors, but the use of these biomarkers in other environmental pollution studies, such as risk assessment or monitoring, is still at an early stage of application and require extensive validation (Neumann and Galvez 2002; Snell et al. 2003; Poynton et al. 2007).

When they were first applied, biochemical biomarkers had a similar status, and much was learned about their use during the last two decades. In the present paper, we reassessed our own published work on three biochemical biomarkers: CAT, GST and ChE in two common test invertebrates: water flea *D. magna* and terrestrial isopod *Porcellio scaber*. We identify some of the characteristics of studied biochemical biomarkers and summarise the lessons learned from their use, thus advancing the application of a new generation of 'omic' biomarkers and identifying possible links between these two groups of biomarkers in future research.

2 Chronological overview of biomarker research

A significant increase in the number of scientific publications containing the keyword 'biomarker' has been observed since the infancy of the discipline at the beginning of the 1980s (Fig. 1). Although encompassing both whole-organism and sublethal responses, the term 'biomarker' refers most commonly to the latter. The biomarker concept was initially applied in medical diagnostics as an indicator of a particular state or disease in humans (Paone et al. 1980), and in the early 1990s, it became very appealing in environmental studies (McCarthy and Shugart 1990; Walker 1992; Depledge and Fossi 1994; Peakall 1994). Between

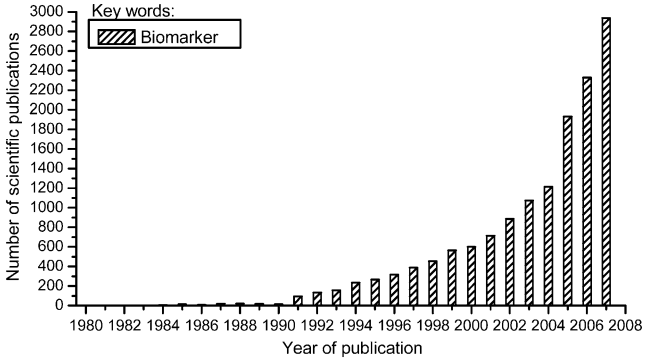

Fig. 1 Number of scientific papers (ISI Web of Knowledge, www.isiknowledge.com) published annually containing keywords biomaker

1990 and 2007, the total number of scientific publications concerning biomarkers has increased by a factor of 200 and currently approaches 3,000 publications annually (see Fig. 1).

At the present time, the number of publications reporting the use of novel techniques, such as genomic and proteomic biomarkers in environmental studies, is increasing. For example, by November 2008, nearly 87 publications were retrieved by the search term of 'genomic and pollution' and 15 for 'proteomic and pollution' (ISI Web of Knowledge, www.isiknowledge.com). However, by our estimations, the number of publications published on the use of these biomarkers in environmental studies is even higher.

3 Early expectations for biochemical biomarkers in environmental pollution studies

At the beginning of biomarker research, there were high expectations for the utilisation of biochemical biomarkers in environmental studies. They were expected to give information on the qualitative and quantitative relationships among chemical exposure, biological response and adverse effects and between biomarker responses and population and community level responses (McCarthy and Shugart 1990). At that time, several criteria were recommended for use of biomarkers in environmental studies. These were defined for all groups of biomarkers, ranging from molecular to the whole-organism performance end-points and also applied to biochemical biomarkers (Walker 1992; Van der Oost et al. 2003) (Table 1).

Several heterogeneous applications of biochemical biomarkers in environmental studies were also suggested (Fig. 2). They were intended either for diagnostic or prognostic purposes, but in reality, following the same diagnostic model as in medical sciences, the use of biomarkers was primarily focused on the assessment of the pollution in the field and monitoring studies and to a much lesser extent in controlled laboratory experiments for prognostic purposes.

4 Case study: intrinsic properties of three prototypical biochemical biomarkers in the two crustacean model species

In the present case study, we investigated the properties of three selected biochemical biomarkers which might affect their application in environmental studies. We re-evaluated our previously published work (Jemec et al. 2007a, b, 2008a, b; Drobne et al. 2008) on three by-far most studied biochemical biomarkers in environmental pollution studies: CAT, GST and ChE (Koce et al. 2007) measured in water flea *D. magna* and terrestrial isopod *P. scaber*.

Three intrinsic properties of CAT, GST and ChE were studied: (a) variability of baseline values in chemically non-stressed crustaceans, (b) their sensitivity, which is related to

Table 1 The criteria for the use of biomarkers in environmental pollution studies proposed by different authors (Depledge and Fossi 1994; Van Gestel and Van Brummelen 1996; Cajaraville et al. 2000; Kammenga et al. 2000; Van der Oost et al. 2003)

Proposed criteria
A link should be established between biomarkers of different organisational levels of an organism (e.g. between sub-cellular and whole-organism levels); e.g. the changes that occur at sub-cellular levels should be indicative of changes observed at the organism levels
Established link between biomarkers of different levels of biological organisation (between organisms and populations, communities, ecosystems); e.g.: the changes that occur at organism level should be followed by changes observed at the population or ecosystem levels
Biomarker should be sensitive to detect early effects-early warning system; e.g. the changes at the sub-cellular level should be detected at lower concentration than those at the whole-organism level
The relation between the biomarker response and exposure levels should be determined; e.g. determine if the response follows a dose response
Clarity/ease of interpretation
Well-understood effects of confounding non-chemical (abiotic, biotic) factors on biomarker response; well known inter-individual variability of biomarker response
The time-dependence of the biomarker response must be understood
Methodological considerations: the biomarker assay should be economic, easy to perform and reliable

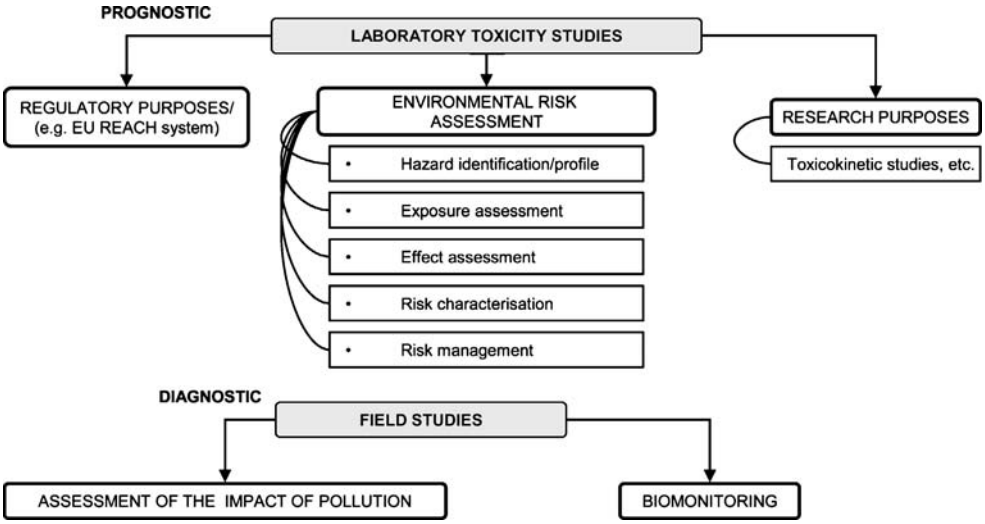

Fig. 2 Suggested applications of biochemical biomarkers in environmental pollution studies (Walker 1992; Depledge and Fossi 1994; Depledge et al. 1995; Handy et al. 2003; Kammenga et al. 2000; Van der Oost et al. 2003)

their mode of action and (c) the link between these biochemical biomarkers and adverse effects at the level of the whole organism. All conclusions presented below refer specifically to the three biochemical biomarkers studied in two selected crustacean species.

4.1 Variability of baseline values of CAT, GST and ChE

We investigated natural environmental factors and endogenous characteristics of the animals as the sources of the variability of CAT, GST and ChE. In the case of the water flea, *D. magna*, marked differences in the baseline enzyme activities of chemically non-stressed specimens measured in different laboratories were observed (Table 2). We attribute these differences to the variability of daphnid clones used and non-standardised laboratory culture conditions used in different laboratories (Barata et al. 2000). Differences can also be attributed to the age of animals, the activities in juveniles being in general higher than in adult daphnids (see Table 2; Printes and Callaghan 2003).

Similarly, high differences between baseline enzyme activities of the chemically non-stressed isopod *P. scaber* and other isopod species were reported by different authors (see Table 2). Because isopods used by different authors were collected at different locations, the differences between baseline enzyme activities can be attributed to various abiotic conditions at the origins of isopods. Specifically, we found that the baseline CAT and GST activities of isopods *P. scaber* depend on the abiotic conditions in the environment (Jemec et al. 2008b). When the isopods were collected from the field and brought to the laboratory, the activities of both enzymes gradually decreased as a result of acclimation of isopods to stable laboratory conditions. Isopods in their natural environment are constantly exposed to various abiotic and biotic factors which result in high variation of antioxidant enzymes like CAT and GST. The effects of natural conditions on the activities of antioxidant enzymes and ChE have been reported previously (Khessiba et al. 2005; Bochetti and Regoli 2006; Monserrat et al. 2007). The changes in activity were attributed to changes in metabolic activity of the organisms as a result of more intensive feeding, different phases of the reproductive cycle (Regoli et al. 2002) and higher temperatures (Abele et al. 1998). On the contrary, no dependence of CAT and GST activities on isopods' endogenous parameters, such as gender, moult stage or presence of brood chamber was found (Jemec et al. 2008b). Similarly, no links between the gender and GST activities were found in other isopods (De Knecht et al. 2001). In other studies, the effect of gender on the CAT and GST activities was found to be species-dependent (Mourente and Díaz-Salvago 1999; Nunes et al. 2004; Vega-Lopez et al. 2007).

In addition, we evaluated the variability of biochemical biomarkers in non-chemically stressed daphnids and isopods measured in our experiments. The coefficients of variation (c.v.) were compared. These values in *D. magna* were: 8–33% (ChE), 9–22% (CAT) and 17–32% (GST) and 63% (CAT) and 42% (GST) in *P. scaber*. The variability of enzyme activities of *D. magna* was lower in comparison to those of *P. scaber*. This is because toxicity tests with daphnids were performed with genetically identical females derived from a single clone of a laboratory culture, and the animals were reared under standard laboratory conditions with a constant supply of food. In contrast, isopods brought from the field were not genetically identical. The literature search also reveals the high variability of biochemical biomarkers in other studies (Mourente and Díaz-Salvago 1999; Olsen et al. 2001; Correia et al. 2003). The factors that need to be considered when measuring biochemical biomarkers are summarised in Table 3.

The variability of baseline enzyme activities observed within one or several different laboratories implies that

Table 2 Specific enzyme activities of baseline (chemically non-stressed) biochemical biomarkers published in the literature

	ChE (nmol/min.mg protein)	Ref.	CAT (μmol/min mg protein)	Ref.	GST (nmol/min mg protein)	Ref.
D. magna	2.5 (1–2 d)	(1)	350 (4–5 d)	(7)	108.4 (3 d)	(5)
	4 (1 d)	(2)	250 (6 d)	(11)	350 (4–5 d)	(7)
	9 (3 d)	(3)	62.4 (22 d)	(9)	200–250 (6 d)	(11)
	8–9 (3 d)	(4)	84.3 (22 d)	(10)	67.3 (22 d)	(9)
	1.24 (3 d)	(5)			87.3 (22 d)	(10)
	3.8 (juveniles)	(6)				
	3.5 (4–5 d)	(7)				
	8 (adult)	(8)				
	3 (7–14 d)	(3)				
	3.5 (21 d)	(3)				
	0.5 (14–21 d)	(2)				
	0.46 (22 d)	(9)				
	0.61 (22 d)	(10)				
P. scaber[a]	2,000 (juvenile, w.b.)	(12)	0–55 (adult, hep.)	(13)	800 (juveniles, w.b.)	(14)
	3,000 (adult, w.b.)	(12)			7,200 (adult)	(15)
					100–900 (adult)	(13)
					1,500 (adults)	(14)
Other isopods[a]	12,000–14,000 (adult, head)	(16)	31–41 (w.b.)	(18)	4,032 (w.b.)	(19)
	9,000–10,000 (juvenile)	(16)				
	5,500–6,500 (adult)	(17)				
Other crustaceans	22	(6)	20	(23)	160–320 [b,c]	(24)
	33–122 [b,c]	(20)	6–68 [b]	(24)	630–1,112 [b,c]	(25)
	0.25	(21)	0.28–49 [b]	(23)	1,200	(26)
	134	(22)			185	(22)

References: (1) Printes and Callaghan 2003; (2) Guilhermino et al. 1996; (3) Diamantino et al. 2000; (4) Guilhermino et al. 2000; (5) Jemec et al. 2007a; (6) Day and Scott 1990; (7) Barata et al. 2007; (8) Diamantino et al. 2003; (9) Jemec et al. 2007b; (10) Jemec et al. 2008a; (11) Barata et al. 2005; (12) Stanek et al. 2006; (13) Jemec et al. 2008b; (14) Drobne et al. 2008; (15) De Knecht et al. 2001; (16) Ribeiro et al. 1999; (17) Engenheiro et al. 2005; (18) Hartenstein 1982; (19) Stenersen et al. 1987; (20) Mora et al. 1999; (21) McLoughlin et al. 2000; (22) Elumalai et al. 2002; (23) Correia et al. 2003; (24) Mourente and Díaz-Salvago 1999; (25) Almar et al. 1987; (26) Livingstone 1998. In cases where the absolute values were not documented, the activities were determined from graphs and therefore represent an approximation. The age of the animals is denoted in parenthesis, unless in cases where it could not be determined. Only GST activities measured with 1-chloro-2,4,-dinitrobenzene as a substrate are provided

hep hepatopancreas, *w.b.* whole body, *d* days

[a] Exact age of field animals cannot be determined

[b] Different species

[c] Different tissues

straightforward combining of experiments is inaccurate. It also happens that the values for control animals vary significantly when repetitions of the same experiments conducted in the same laboratory are compared. To avoid these problems, different sets of experiments can be combined when toxicity values are expressed as a relative measure, for example as percentages of control values (Jemec et al. 2008a).

4.2 Sensitivity of CAT, GST and ChE in comparison to whole-organism responses of the two crustacean species

Biochemical biomarkers are generally considered to be more sensitive than whole-organism responses, which means that the changes at the sub-cellular level should be detected at lower concentrations than the ones at the whole-organism level. To evaluate the sensitivity of selected enzyme biomarkers, we compared the lowest observed effect concentration values (LOEC) of biochemical biomarkers to the LOEC values obtained for whole-organism responses. All toxicity data for daphnids and isopods are shown in Tables 4 and 5, respectively.

From the results obtained for both test organisms, we conclude that selected enzyme activities in these two crustaceans are not always more sensitive than higher level end-points. Their sensitivity depends on the mode of action of a chemical and is duration- and species-specific.

Table 3 Factors that need to be considered when measuring biochemical biomarkers

Factors to be considered	Specific description
1. Origin of test specimens	Natural conditions at the site of collection
2. Maintenance of specimens in the laboratory	Abiotic conditions of culturing in the laboratory
	Feeding regime
	Period of maintenance in the laboratory prior to toxicity tests
3. Endogenous characteristics of the specimens	Gender
	Age
	Sexual maturity
	Reproduction stage
	Moult stage
	Fitness of test animals (presence of infections)
4. Characteristics of test species	Physiology
	Anatomy
	Ecology
5. Optimised methodology for enzyme measurements	Optimal pH, temperature, concentrations of substrates
	Optimal concentration of proteins
	Removal of excess pollutants in the homogeniser to prevent in vitro effect on enzymes (in case of daphnids)
	Determine the source of enzymes (whole body, specific organ,...)
6. Interpretation of data	Appropriate reference for the calculation of specific enzyme activities (protein content, animal weight, etc....)
7. Final information outcome	Integration of all knowledge and obtained data on test specimens (points 1–6)

Similarly has been also been reported for other species (Sturm and Hansen 1999; McLoughlin et al. 2000; Brown et al 2004b). It has also been suggested that the high variability in the responsiveness of biochemical biomarkers is a function of the species being investigated, the periods of exposure and the class of chemicals (Livingstone et al. 1995; Regoli et al. 2002).

4.3 Link between tested biochemical biomarkers and adverse effects at the organism level

One of the suggested criteria for the use of biochemical biomarkers in environmental pollution studies is that they should exhibit a link to adverse effects at the organism level including processes such as growth, reproduction and mortality (Depledge and Fossi 1994). Our results show that certain changes of tested enzymes in the two crustaceans are preceded by effects at a higher level of biological organisation, but this may not be generally true (see Tables 4 and 5). Much other published work also suggests that the link between sub-lethal biomarkers and whole-organism responses is not always clear. For example, the assumption was made that the 50% inhibition of ChE is indicative of a life-threatening situation (Ludke et al. 1974). However, Printes and Callaghan (2004) observed no immobility of *D. magna* exposed to 100 μM of the organophosphate acephate, although ChE activity was inhibited by 70% in such cases as compared to the control. On the other hand, no effects of organophosphate (OP) on ChE activity were observed in the stonefly *Claassenia* sp. exposed to near-lethal concentrations of fenitrothion (Day and Scott 1990). Keizer et al. (1995) showed that the toxicity of organophosphate is species-dependent and depends on its rate of bioactivation by conversion to the more potent oxon form, detoxification in the organism and the affinity of ChE for a organophosphate. In cases where ChE remains unchanged but mortality of animals occurs, other mechanisms of OP toxicity besides ChE inhibition are probably involved.

4.4 Methodological considerations and interpretation of data

Utilisation of biochemical biomarkers in environmental studies anticipated relatively easy measurements and easy interpretation of data. Based on our experience, we consider the measurements of biochemical biomarkers in *D. magna* and *P. scaber* demanding because a number of steps have to be optimised for each enzyme and for each organism separately to obtain reproducible and reliable results (Jemec et al. 2007a). The main limitation of enzyme assays is the total amount of proteins needed. Consequently, measurements of enzyme activities in individual daphnids or individual tubes of digestive glands of isopods are not

Table 4 LOEC and NOEC values obtained for daphnids exposed to two metals (Cr, Cd) and two pesticides (diazinon, imidacloprid; Jemec et al. 2007a,b, 2008a,b)

	Short-term exposure (48h)				Long-term exposure (21days)			
	[a]Cr^{6+}	[b]Cd^{2+}	Diazinon	IMI	[a]Cr^{6+}	[b]Cd^{2+}	Diazinon	IMI
Highest concentration tested	280	40	7	40,000	52.5	2.62	8	40,000
LOEC (μg/L)								
Biochemical biomarkers	>280[c]	>40[c]	>7[c]	>40,000[c]	1.1	0.032	5<×<8	1,250
Whole-organism responses	250	30	5	20,000	>52.5[c]	0.656	8	2,500
Combined (bio. + whole-organism)	250	30	5	20,000	1.1	0.032	5<×<8	1,250
NOEC (μg/L)								
Biochemical biomarkers	280	40	7	40,000	0.52	0.041	5	650
Whole-organism responses	210	25	4	10,000	35	0.328	5	1,250
Combined (bio. + whole-organism)	210	25	4	10,000	0.52	0.041	5	650
Toxicity ranking LOEC/NOEC								
Biochemical biomarkers	n.d.				Cd^{2+}< Cr^{6+}< diazinon < IMI			
Whole-organism responses	diazinon<Cd^{2+}<Cr^{6+}< IMI				Cd^{2+}< diazinon < IMI; Cr^{6+} [d]			
Combined (bio. + whole-organism)	diazinon<Cd^{2+}<Cr^{6+}< IMI				Cd^{2+}< Cr^{6+}< diazinon < IMI			

The LOEC/NOECs were determined for all biochemical biomarkers together (ChE, CAT and GST activity) and together for whole-organism responses (immobility and reproduction). The LOEC/NOECs were also determined for all parameters tested (combined biochemical + whole-organism)

IMI imidacloprid, *LOEC* lowest-observed effect concentration, *NOEC* no-observed effect concentration, *x* the LOEC or NOEC value is between the two values, *n.d.* ranking could not be determined

[a] ($K_2Cr_2O_7$)

[b] ($CdCl_2$)

[c] > the LOEC was not observed up to the highest concentration tested

[d] Ranking could not be determined

possible. The measurements are particularly time-consuming in case of daphnids because a large number of neonates (up to 800) of the same age and derived from the second to fifth brood are needed in these tests. Additionally, we found that the excess chemical must be rinsed from the surface of the animals and homogeniser to avoid possible in vitro effects of excess chemical on the enzyme activities. It also turned out that it is important to consider which organ is used for enzyme measurements. In case of isopods, there are significant differences in the variability of baseline values if the whole body is measured or only the digestive glands (hepatopancreas). Namely, the whole body also contains gut, which is either empty or full and consequently contains different amounts and populations of gut bacteria. Our unpublished data suggest that gut bacteria contribute to the total CAT activity when the whole body is used. Due to all these reasons, we find measurements of selected biochemical biomarkers in *D. magna* and *P. scaber* not well suited to routine use, in particular at wastewater plants or industrial laboratories using *D. magna* as a test species.

Accurate interpretation of data derived from such studies requires background knowledge in biochemistry, test organism physiology and toxicology. Special attention must be given to the expression of enzyme activities per protein content, since it can be changed due to chemical exposure (Knowles and McKee 1987). Possible misinterpretation of enzyme activities using proteins as a reference was pointed out in our work (Jemec et al. 2007b, 2008a) and has also been reported by others (Radenac et al. 1998; Brown et al. 2004a).

In summary, biochemical biomarkers in *D. magna* and *P. scaber* reviewed in the present work failed to fulfil the majority of criteria initially articulated for their use in environmental studies (see Table 1).

5 Discussion, recommendations and perspectives

In invertebrates, the three commonly studied biochemical biomarkers of CAT, GST and ChE have been successfully

Table 5 LOEC and NOEC values obtained for adult isopods exposed to two metals (Cr, Cd) and two pesticides (diazinon, imidacloprid) (Drobne et al. 2008; Stanek et al. 2006)

	Short-term exposure (3days)			Long-term exposure (14days)	
	Cd^{2+}($CdCl_2$)	Zn^{2+}($ZnCl_2$)	Cu^{2+} ($CuCl_2$)	Diazinon	IMI
Highest concentration tested	500	5,000	5,000	100	25
LOEC (μg/L)					
Biochemical biomarkers	100	1,000	>5,000[a]	50	25
whole-organism responses	>500[a]	5,000	5,000	100	10
Combined (bio. + whole-organism)	100	1,000	5,000	50	10
NOEC (μg/L)					
Biochemical biomarkers	10	<1,000	5,000	10	10
Whole-organism responses	500	2,000	2,000	50	<10
Combined (bio. + whole-organism)	10	<1,000	2,000	10	<10
Toxicity ranking LOEC					
Biochemical biomarkers	Cd^{2+}< Zn^{2+}< Cu^{2+}			IMI < diazinon	
whole-organism responses	Cd^{2+} [b], Zn^{2+} = Cu^{2+}			IMI < diazinon	
Combined (bio. + whole-organism)	Cd^{2+}< Zn^{2+} < Cu^{2+}			IMI < diazinon	
Toxicity Ranking NOEC					
Biochemical biomarkers	Cd^{2+} < Zn^{2+} < Cu^{2+}			IMI = diazinon	
Whole-organism responses	Cd^{2+} < Zn^{2+} = Cu^{2+}			IMI < diazinon	
Combined (bio. + whole-organism)	Cd^{2+} < Zn^{2+} < Cu^{2+}			IMI = diazinon	

The LOEC/NOECs were determined for all biochemical biomarkers together (ChE, CAT and GST activity) and together for whole-organism responses (mortality and feeding). Also the LOEC/NOECs were determined for all parameters tested (combined biochemical + whole-organism)

IMI imidacloprid, *LOEC* lowest-observed effect concentration, *NOEC* no-observed effect concentration

[a] > the LOEC was not observed up to the highest concentration tested

[b] Ranking could not be determined

used as diagnostic tools in field studies (Regoli et al. 1998; Khessiba et al. 2005; Demásio et al. 2007; Markert 2007; Minutoli et al. 2007) and in laboratory toxicity studies for generation and testing of specific hypotheses concerning mechanisms of chemical impact at different levels of organisation (Forbes et al. 2006). However, they have not been sufficiently exploited either in environmental risk assessment (ERA) or for purposes of chemical regulation. To advance their use in such applications, we suggest based on their properties that their role should be more specifically defined. Namely, ERA includes several distinct steps with different purposes. In the first step, adverse effects, which a substance has an inherent capacity to cause, are identified (hazard identification step). Afterwards, the relationship between dose of exposure to a substance and an effect are estimated (effect assessment), and potential environmental exposure levels are estimated (exposure assessment). Finally, risk characterisation is performed by estimation of the incidence and severity of the adverse effects likely to occur in environment (i.e. the quantification of that likelihood; TGD Document 2003). For the last step, a standardised base set of required toxicity data has been defined, but hazard identification, a step whose purpose is to provide a holistic view of the potential hazard of the substance in question, is more flexible in terms of using different tools. In hazard identification, it is crucial to have as much information as possible on the effects at different levels of biological organisation (Van der Oost et al. 2003). Based on the intrinsic properties of biochemical biomarkers identified in the present study, we suggest that they are well suited to hazard identification but are much less appropriate for risk characterisation. Successful application of biomarkers in ERA depends on continuing and effective communication between the risk assessors and scientists in order to reach a consensus between hazard assessment, the ‘demand-side’ and biomarker research, the ‘supply-side’.

In the scope of hazard identification, biochemical biomarkers could be used to rank chemicals according to their hazardous potential (see Tables 4 and 5). This provides comparative toxicity data, which are very useful for further direction of toxicity investigations. We discovered that the tested enzyme activities are not always more sensitive than whole-organism responses, as was anticipated, their sensitivity depending on the mode, duration and test organism species. Therefore, we suggest that a

combination of a battery of biomarkers from different levels of biological complexity and also an array of biomarkers within a single level could identify hazard adequately. The use of a range of biochemical biomarkers involved in different metabolic processes could reduce false positive or false negative hazard assessments.

Two decades ago, biochemical biomarkers were considered to be a 'new powerful approach' (Depledge et al. 1995), a 'diagnostic tool for individual health', a 'predictive tool for changes at population level' (Lagadic 1999) and a 'logical approach to ERA which has already proven its worth' (Walker 1999). Similar expectations have been recently expressed for '-omics' approaches (Benninghoff 2007; Mi et al. 2007; Chora et al. 2008; Poynton et al. 2008), although some limitations and a need for further validation have been discussed (Neumann and Galvez 2002). For example, precise investigation of background variation expression profile unrelated to the contaminants is necessary. A normal response of an organism to retain homeostasis has to be distinguished from the response of stressed or adversely affected organism (Calzolai et al. 2007). In order to facilitate the application of 'omic' biomarkers in environmental studies, we suggest that the substantial body of experience obtained with biochemical biomarkers should be exploited in the development of new-generation biomarkers.

The lesson learnt concerning the biomarkers evaluated in the present review is that for successful application, it is of crucial importance to understand their intrinsic properties before an (eco)toxicity study is designed. The health status of the test organism and the sources of biomarker variability, such as gravidity, moulting, bacterial infection or parasites should be known. These factors are commonly overlooked in invertebrate biomarker studies.

In the future, the application of biomarkers in environmental studies will require a combination of both traditional, e.g. biochemical, and new-generation 'omic' biomarkers. For research purposes, complete ecotoxicity information should include contributions from the molecular fingerprint revealed by the use of 'omic' techniques to the whole-organism responses. However, in routine use, the group of biomarkers applied will probably depend on their reproducibility, ease of use, robustness and affordability of the methodology as well as the type of chemicals, organisms and ecosystem of interest. With the use of data obtained by transcriptomic/proteomic tools, it is possible to identify entire groups of genes and proteins involved in stress response and in such a way acquire new knowledge which might encourage again the development and use of traditional types of biomarkers (e.g. biochemical, cellular, histological, physiological, etc.).

In conclusion, the past experiences gained on biochemical biomarkers in environmental pollution studies should be exploited to new-generation '-omics' biomarkers. The future of biomarker research lies in combining the knowledge of both traditional and new generations of biomarkers.

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