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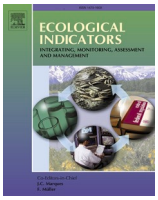

Review

Enzymes as useful biomarkers to assess the response of freshwater communities to pesticide exposure – A review

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ABSTRACT

The lack of specificity of pesticides used for control of various organisms, even if efforts have been made to design formulations more species-specific, produce harmful effects on non-target organisms. Oxidative stress and lipid peroxidation are amongst the main known effects induced by pesticide exposure, besides eventual lethal toxicity, endangering organisms' biomembranes integrity or compromising their activity through long-term exposure. Due to the general persistency of pesticides in the environment, these can easily be transported by water runoff. Pesticides used in agricultural fields are frequently transported to nearby freshwater systems, potentially affecting non-target organisms. Organophosphorus pesticides and carbamates are amongst the widely used classes of pesticides, and, despite being considered of rapid biodegradation, present a broad-spectrum of action, and the frequent transport of these contaminants to other systems may be harmful to non-intended species. Others, such as organochlorine pesticides, are highly persistent in the environment, posing a threat to non-target species for long periods. This is a matter of utter importance given that pesticides are known to impair numerous biological processes and inhibit the action of key enzymes in the response to xenobiotic-induced stress, potentiating oxidative stress and neurotoxicity, with potential irreversible effects. The study of the effect of pesticides has for long assessed exposure responses in a set of antioxidant and esterase enzymes, along trophic levels. The information is, however, more vast concerning photosynthetic organisms, macroinvertebrates and fish, with zooplankton appearing to be the group least studied. Given the ecological importance of zooplankton, further information regarding the response of this group to pesticide exposure could help detect early warning signs of potential threats to an ecosystem's integrity and search for alternatives and solutions to prevent the harmful action of pesticides in non-target individuals, that escalate food chains. Nonetheless, the use of enzymes as biomarkers to assess the response of freshwater communities to stress induced by pesticides has for long proven to be an effective tool. Some constraints related to the consistency of organisms' sensitivity and responses to pollutants may, however, lead to results not always straightforward, as a same pesticide may produce different enzymatic responses depending on the organism affected or to the environmental conditions. Thus, further studies using enzymes as biomarkers of pesticide exposure could provide more information and understanding to overcome the existing limitations and strengthen the applicability of enzymes in this context.

1. Introduction

The continuous and increasing worldwide use of pesticides to prevent proliferation of unwanted species in agriculture fields or industrial plants, which may potentially compromise full production, has been leading to the contamination of terrestrial and aquatic ecosystems (both surface and ground waters). Aquatic systems are particularly affected by such substances, that decrease water quality and impact non-target species, regardless of trophic level, potentially disrupting the ecological balance of the environments (Moraes et al., 2007). Organisms' metabolic and biochemical processes, as well as regulatory mechanisms, may be disturbed, including changes in organisms' energy metabolism (Villarroel et al., 2009), neurotransmission impairment (Chebbi and David, 2009) and oxidative stress. The latter two are the most commonly studied toxicological mechanisms induced in organisms by exposure to pesticides. Such parameters become of high biological and ecological relevance particularly in aquatic ecosystems, as these environments may accumulate various contaminants from several

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sources that potentially induce oxidative stress in organisms (Kelly et al., 1998).

Exposure to stressors generally induces changes in the normal repartition of organisms' energy. Most organisms in non-stress conditions allocate energy for basal metabolism, growth and reproduction. In stress conditions, for example caused by toxicant exposure, organisms' energy allocation is changed in order to cope with the induced stress (Bendis and Relyea, 2014; Jeon et al., 2013), resulting in a decrease in energy reserves and transfer of energy outflows to a single mechanism among the above-mentioned (Sancho et al., 2009), in an effort to guarantee the individual or the species' survival.

Oxidative stress induced by pesticides, through an increase in the formation of reactive oxygen species (ROS), may lead to biochemical, cellular and physiological changes in the exposed organisms. Free radicals may lead to lipid peroxidation, tampering with biological membranes' constitution, as well as oxidative damage to DNA and proteins (Kelly et al., 1998). Nonetheless, cells of every living organism have protective mechanisms against oxidative stress, aimed at balancing the redox status and maintaining cell homeostasis against ROS, a process that may be mediated by antioxidant enzymes. The most important enzymes involved in these processes are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Oruç and Usta, 2007).

Given the variety of toxic effects that can be generated by pesticide exposure, various biomarkers to measure organisms' responses to such contaminants may be applied The sensitivity and specificity of biomarkers vary depending on the contaminant, or group of contaminants, existing biomarkers more suitable to different sets of contaminants. Biomarkers to be used as endpoints in ecotoxicology may be suitable to detect the presence of particular contaminant(s) and presenting high sensitivity to the compound(s) (allow early detection, responding to low contaminant concentration) (Bennett and Devarajan, 2011; Strimbu and Tavel, 2010). Thus, biochemical biomarkers are being considered of great toxicological relevance due to the fast response presented to tests of sublethal contamination doses at the lowest biological organization levels, i.e. biochemical/cellular responses. They are an emerging means to assess the extent of the damage induced in organisms by contaminants, and determine how these affect organisms' survival, as well as providing information about effects at higher levels of biological organization.

Over the years, numerous pesticides have been developed to be applied in bare environments (Aktar et al., 2009; Lazartigues et al., 2013; Margni et al., 2002; Maugh, 1978). Most products are mixtures of active substances – chemical compounds that are biologically active and produce the wanted effect - with other compounds, as facilitators of spreading or bioaccumulation of the active substance. By exploring different structures and properties, pesticides may be designed to affect different targets and have different action mechanisms. Classification of pesticides may be according to the target-group (herbicides, insecticides, fungicides, bactericides, etc.) or chemical class.

Organophosphorus pesticides (OPPs), such as parathion, chlorpyrifos (CP), dichlorvos or azinphos methyl (AZM), and carbamates (CMs), such as molinate, carbaryl or carbofuran, are within the most common and widely used pesticides. Although some OPPs and CMs present rapid biodegradation if applied correctly, the wide use, runoff events and the high toxicity of some of these products present to unintended species cause a serious concern about their potential deleterious ecological impacts. These substances are inhibitors of cholinesterase enzymes (e.g. acetylcholinesterase and butyrylcholinesterase), causing neurotoxicity to numerous non-target organisms, such as insects, birds, mammals and fish, as these possess the enzymes targeted by the pesticides (Johnson, 1990; Mineau, 1991; Nunes, 2011; Wang and Murphy, 1982).

Organochlorine pesticides (OCPs) are the most toxic and persistent pesticides in the environment. This class of pesticides includes aldrin, chlordane, heptachlor, hexachlorobenzene, alpha and beta hexachlorocyclohexane, lindane, mirex, toxaphene and dichlorodiphenyltrichloroethane (DDT) (Sankararamakrishnan et al., 2005), to name some examples, all with the proper,ty of bioaccumulating in biological membranes and magnifying along food chains (Van Dyk and Pletschke, 2011).

Triazine pesticides are a fourth class of pesticides to be taken into consideration, consisting predominantly of herbicides able to selectively inhibit the electron transport in photosynthesis, thus representing a serious concern about the potential effect on non-target photosynthetic organisms.

Glyphosate-based herbicides are amongst the most common pesticides used in weed control and act at various levels, namely impairing the synthesis of aromatic aminoacids, affecting photosynthesis, mineral nutrition, and provoking oxidative stress (Helander et al., 2012; Gomes et al., 2016; Miteva et al., 2010). Moreover, the broad use of glyphosate from industrial to household pesticides has recently drawn more attention to the potential harmful effects of the substance to humans and other animals.

Other pesticide classes, such as neonicotinoid and organochlorine (OC) pesticides, include many other substances to be taken into consideration regarding the nefarious consequences they pose to non-target species. Dichlorodiphenyltrichloroethane, commonly known as DDT, for example, belongs to the OC class of pesticides. This substance gained particular interest due to its very high persistency in the environment, as well as the toxic effects it poses to non-target organisms, namely to human health, which led to DDT's eventual ban from overall agricultural practices worldwide, although it is still used in particular situations (Thakur and Pathania, 2020).

Over the years, especially in more developed countries, highly toxic and persistent pesticides have been legally banned, such as the previously mentioned DDT, and replaced with others faster degrading and more species-specific. However, mainly in developing countries, the low cost/efficiency ratios of some more toxic pesticides rule over the potential ecological impacts they pose, sometimes leading to the use of banned substances. Even more recent pesticides, although presenting improvements in terms of ecological damage, still pose serious ecotoxicological issues. Therefore, there is an increasing need to understand the processes that generate such effects and to what extent they may affect non-target organisms.

For the past decades, numerous studies have evaluated the response of aquatic organisms to pesticide contamination, through the evaluation of biomarkers, as mentioned elsewhere (*e.g.* Galhano et al., 2011b, 2011a; Rosas et al., 1980). Biomarkers are measurable biological parameters that indicate some sort of change in a considered biological system (NRC, 1987; van der Oost et al., 2003).

The ecotoxicological effect of pesticides in non-target species, regarding the species' response to contaminants, is often unknown and difficult to predict, as these compounds were not designed to affect such organisms, but are highly likely to do so. For this reason, it is of extreme importance to assess the response of those organisms in physiological and biochemical terms to the toxicants. However, the choice of the right biomarker is not always simple, as ecotoxicity mechanisms are rarely fully understood. The current challenge is, then, to choose or define reproducible and accurate biomarkers that reveal the outcome of potentially harmful effects of different types of pesticides in different organisms.

2. Current trends in using enzymes as biomarkers of non-target organisms' response to pesticides

The present work aims to critically review available literature on the use of enzymes to assess the response of freshwater organisms to pesticide exposure in the environment. The publications selected to produce this literature review analyzed the response of non-target freshwater communities, across trophic webs, to contaminant exposure.

This critical review underlines the broad use of enzymes as biomarkers to identify and assess the response of freshwater organisms to pesticides present in the environment. Nonetheless, the work

emphasizes the constraints and limitations of the use of a single biomarker to determine such responses, as organisms may react differently to a same contaminant, possibly influenced by other surrounding or intrinsic factors. Moreover, the literature review notes the lack of information concerning the use of enzymes as biomarkers to assess responses of zooplankton communities to stressors, comparing to other groups of different trophic levels, especially given the ecological importance of the group. Overall, the use of enzymes as biomarkers of contaminant impacts in non-target species has proven to be a useful tool to assess the impacts in non-target species. Nonetheless, further steps may still be taken in terms of analysis or application methods to surpass some constraints that prevent enzyme assessment from being an even more reliable and reproducible biomarker, as mentioned throughout the critical review that follows.

3. Enzymes as suitable biomarkers of pesticide contamination

Enzymes are among the most widely used biomarkers to assess the response of organisms to toxicants, providing essential clues to predict the impact of the chemicals in both target and non-target organisms.

As previously stated, OPPs and CMs inhibit the activity of cholinesterase enzymes (ChE), complex enzymes with many forms and functions belonging to the class of esterases, although not all esterases interact or are inhibited by those classes of pesticides (Basack et al., 1998). It is worth noticing that the interpretation of results from studies assessing changes in ChE is not always simple, as enzymatic responses are not necessarily straightforward or predictable. In fact, inhibition of ChE activity after xenobiotic exposure may not immediately mean an impairment of the enzyme's activity due to the contaminant; rather, the activation of cells' compensatory mechanisms also need to be considered, which fight anti-cholinesterase compounds and remediate their harmful effects, potentially resulting in a decrease of ChE activity. These natural defense mechanisms may influence the enzymes' activity and mask the actual effect of the xenobiotic on ChE, posing a constraint in the use of ChE as biomarkers.

Nevertheless, cholinesterase enzymes have been frequently used as biomarkers to evaluate the ecotoxicological effects of many OPPs, CMs and other contaminants, due to their sensitivity to a high number of pesticides, the low costs associated to the analysis, simple quantification analysis, reproducibility and biological and ecological relevance (Nunes, 2011).

Acetylcholinesterase (AChE) is mainly present in postsynaptic neurons and catalyzes the hydrolysis of the neurotransmitter acetylcholine, terminating the nervous impulse and allowing the cell to return to its resting state. AChE is also relevant in the differentiation and apoptosis of nerve cells. Some pesticides interact with AChE's active site, as OPPs, inhibiting its activity and causing an accumulation of the enzyme in the synaptic cleft, potentially leading to behavioral changes, paralysis and even death (Jeon et al., 2013). Butyrylcholinesterase (BChE) is mainly present in the plasma of most vertebrate organisms. This enzyme is thought to play a role in the regulation of cell proliferation and neuron differentiation (Nunes, 2011). Carboxylesterases (CbEs) work as catalyzers of hydrolytic reactions of chemicals, also serving as protectors of AChE in organisms. CbEs are very sensitive to OPPs and CMs, and their inhibition by pesticides is considered a detoxification mechanism, that does not produce any apparent negative effect in the organism directly related to the inhibition of CbEs, while avoiding the inhibition of AChE (Van Dyk and Pletschke, 2011). CbEs' inhibition may, then, also indicate a response of organisms to pesticides, as a detoxification mechanism had to be activated, and further impacts may be suspected. The higher sensitivity of CbEs to OPPs and CMs, when compared with ChE's sensitivity, especially in invertebrates, where these enzymes seem to exist in higher quantities, is another aspect in favor of the use of this class as biomarkers to assess pesticide exposure responses. Moreover, if both biomarkers are combined, a more complete understanding of the toxic mechanisms and responses may be reached (Kristoff et al., 2010).

The generation ROS and consequent oxidative stress is an almost ubiquitous effect induced by pesticide exposure in non-target species, verified by many authors over the years (Sayeed et al., 2003; Toni et al., 2013; Kavitha and Rao, 2008; de Menezes et al., 2012). ROS are produced in all organisms' cells in normal conditions, mainly in mitochondria, chloroplasts and peroxisomes, and are needed for certain cell functions. However, production and elimination rates of ROS must be in balance to guarantee cell redox homeostasis. This balance is preserved by enzymes and molecules jointly referred to as antioxidant, given their ability to protect cells' molecules and organelles from oxidation (Dröge, 2002). Superoxide dismutase (SOD), catalase (CAT) and glutathione-based enzymes, as glutathione reductase (GR) and glutathione peroxidase (GPx), are the main enzymatic antioxidant defenses of the cell (Apel and Hirt, 2004). SOD – which converts the superoxide anion radical ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2) – is considered the cell's first protective shield against the oxidative effects of superoxides. CAT – which detoxifies H_2O_2 to molecular oxygen (O_2), through the reaction of the enzyme's porphyrin heme groups with H_2O_2 – is an enzyme present in almost all organisms (Oruç and Usta, 2007). GPx, which also detoxifies hydroperoxides, and GR, are the two enzymes responsible for the glutathione (GSH) reduction oxidation cycle. GSH is a tripeptide possessing thiol groups, serving as a significative antioxidant of most organisms' cells, protecting them from damages caused by ROS. In the presence of H_2O_2, for example, GSH is oxidized to glutathione disulfide (GSSG), a reaction catalyzed by GPx. GSSG can then be reduced again into the GSH form, to continue acting as an electron donor to unstable, dangerous molecules, a reduction catalyzed by GR (Pompella et al., 2003). Ascorbate peroxidases (APx) are another family of detoxifying enzymes that react with peroxides reducing them to water (Noctor and Foyer, 1998). Glutathione S-transferases (GST) are a family of enzymes that sustain an important detoxification mechanism, by catalyzing the binding of GSH to xenobiotics that are possibly harmful to the cells, turning them into more water-soluble molecules to facilitate their expulsion (Eaton and Bammler, 1999).

When a contaminant induces oxidative stress, the balance between ROS generation and elimination is disrupted, due to an abnormal production of ROS or by the impairment of the activity of one or more antioxidant enzymes. In response to this stress, the cell will increase the activity of its antioxidant mechanisms and try to reestablish homeostasis. However, if the stress is too high or too persistent, the antioxidant response will not be able to fully compensate ROS overproduction (Dröge, 2002).

4. Enzymatic activity changes in freshwater organisms exposed to pesticides

Various studies throughout the years show impacts in the activity of a number of enzymes in response to exposure to environmental toxicants, from the stimulation of antioxidant enzymes in an effort to reestablish redox balance and preserve cells integrity, to the disruption of enzyme-mediated processes. In the following sections, a review of the studies conducted on this subject in the past decades is presented, highlighting the main results found concerning the response of organisms with different biological complexity to pesticide exposure, through the assessment of enzymatic biomarkers.

4.1. Photosynthetic organisms

Photosynthetic organisms, especially microalgae, have been widely used for ecotoxicology assessment studies, due to their high sensitivity and ecological relevance, as they stand on the basis of aquatic trophic chains as primary producers (Ma et al., 2006). Thus, harmful effects of pesticides on these organisms may affect whole food webs, endangering multiple non-target organisms (Esperanza et al., 2016; Martinez et al., 2015; Rioboo et al., 2007; Romero et al., 2011).

Herbicides are widely used to control the unwanted growth of weeds

Table 1
Effects of pesticides in enzymatic activity of photosynthetic organisms. When the value of the lowest concentration used in the mentioned studies is not specified in the article, the notation "*" is used to express that value.

	Species	Pesticide	Concentration Range	Max. Exposure Period	Studied Enzyme	Lowest effect concentration	% Activity (in relation to control)	Reference
OPP	*Nostoc muscorum*	Bentazon	[0.75; 2] mM	72 h	SOD	0.75 mM	+ 26	Galhano et al., 2011a
					CAT		+ 29	
					APx		+ 41	
					GR		− 33	
					GST		+ 33	
	Chlorella pyrenoidosa	Chlorpyrifos	[2.4 × 10^3; 38.4 × 10^3] µg.l^{-1}	72 h	SOD	2.4 × 10^3 µg.l^{-1} (24 h)	increase	Chen et al., 2016
				72 h	CAT		increase	
	Merismopedia sp.				SOD	4.8 × 10^3 µg.l^{-1} (24 h)	increase	
					CAT		increase	
						3 × 10^3 µg.l^{-1} (24 h)		
						3 × 10^3 µg.l^{-1} (24 h)		
	Chlorella vulgaris	Diazinon	[0.5;5,20,40,100] mg. l-1	12 days	SOD	5 (12 h)	Increase	Kurade et al., 2016
					CAT	20(12 h)	increase	
						40 (12 h)	increase	
						100 (12 h)	increase	
						20 (6 h)	increase	
						100 (12 h)	increase	
	Chroococcus turgidus	Chlorpyrifos	6 × 10^3 µg.l^{-1}	48 h	SOD	6 × 10^3 µg.l^{-1}	+ 48	Kumar et al., 2014
					CAT	6 × 10^3 µg.l^{-1}	+ 81	
	Phytoplankton community	Glyphosate	[0.1; 1000] µg.ml^{-1}	96 h	SOD	1000 µg.ml^{-1}	increase	Smedbol et al., 2018
					CAT	1000 µg.ml^{-1}	increase	
					APX	500 µg.ml^{-1}		
CMs	*Nostoc muscorum*	Molinate	[0.75; 2] mM	72 h	SOD	0.75 mM	− 34	Galhano et al., 2011b
					CAT		− 25	
					APx		− 70	
					GR		− 84	
					GST		− 8	
					GSH		−92	
					GSSG		−98	
OCPs	*Nostoc muscorum*	Endosulfan	[2.5;15]µg.ml^{-1}	20 days	SOD	*	increase	Kumar et al., 2008
	Aulosira fertilissima				CAT		increase	
	Anabaena variabilis				APx		increase	
					SOD	*	increase	
					CAT		increase	
					APx		increase	
					SOD	*	increase	
					CAT		increase	
					APx		increase	
Others	*Scenedesmus obliquus*	Fenhexamid (anilide - fungicide)	[25;100] µg.l^{-1}	96 h	GR	25 µg.l^{-1}	≈ + 57	Mofeed and Mosleh, 2013
					CAT		≈ + 39	
					GST		≈ + 25	
		Atrazine (triazine - herbicide)	[25;100] µg.l^{-1}		GR	25 µg.l^{-1}	≈ + 83	
					CAT		≈ + 111	
					GST		≈ + 47	
		Oxyfluorfen (diphenyl ether – herbicide)	[2.07 × 10^{-5}; 6.22 × 10^{-5}] mM	24 h	GR	4.15 × 10^{-5} mM	+ 47	Geoffroy et al., 2002
					CAT	2.07 × 10^{-5} mM	+ 36	
					GST	6.22 × 10^{-5} mM	+ 76	
					APx	4.15 × 10^{-5} mM	+ 29	
		Diuron (phenylurea – herbicide)	[2.14 × 10^{-5}; 6.44 × 10^{-4}] mM		GR	4.29 × 10^{-5}* mM	+ 46	
					CAT	6.44 × 10^{-5} mM	≈ − 15	
					GST	4.29 × 10^{-5}* mM	≈ + 33	
					APx	*No significant changes at all concentrations*		
		Lactofen (diphenyl ether – herbicide)	[0.1;1] µg.l^{-1}	96 h	CAT	0.1 µg.l^{-1}	increase	Cheng et al., 2015
					SOD	0.1 µg.l^{-1}	increase	
		Desenthyl lactofen (lactofen metabolite)	[0.1; 1] µg.l^{-1}		CAT	1 µg.l^{-1}	increase	
					SOD	0.5 µg.l^{-1}	increase	
		Acilfluorfen (lactofen metabolite)	[0.1; 1] x10^3 µg.l^{-1}		CAT	0.1 × 10^3 µg.l^{-1}	increase	
					SOD	0.5 × 10^3 µg.l^{-1}	increase	
		Flumioxazin	3 µg.l^{-1}	48 h	CAT	3 µg.l^{-1}	+ 37 (6 h)	Geoffroy et al., 2004
					GR		+ 10 (6 h)	
					APO		+ 16 (6 h)	
	Lemna minor	Diuron (phenylurea – herbicide)	100 µg.l^{-1}	96 h	APO	*No significant changes found*		Teisseire and Vernet, 2000
					P-POD	*No significant changes found*		
					G-POD	100 µg.l^{-1}	≈ + 112 (12 h)	
					GR	100 µg.l^{-1}	≈ + 117 (12 h)	
					CAT	100 µg.l^{-1}	≈ − 30	
					GST	100 µg.l^{-1}	≈ + 10 (24 h)	
			3.3 × 10^{-2} mM	96 h		3.3 × 10^{-2} mM		

(*continued on next page*)

in agricultural fields, especially during field preparation for sowing, targeting mostly the photosynthesis of the species. Due to this, all primary producers are potentially impacted by herbicides, both target and non-target organisms. Moreover, as pesticide runoff from agricultural fields is practically impossible to fully contain, different aquatic environments and their trophic webs are potentially exposed to the contaminants.

There is fairly vast available information regarding the response of various photosynthetic species to exposure to different herbicides. Some of the most commonly studied species are the cyanobacteria *Nostoc muscorum, Aulosira fertilissima, Anabaena variabilis,* the microalgae *Scenedesmus obliquus* and *Chlamydomonas reinhardtii.*

Molinate and bentazon are two commonly used active agents of commercial herbicide formulations (as Ordram® and Laddok®, respectively) used to control weed growth in rice fields, that impair photosynthesis by inhibiting the electron transport of photosystem II. Their effect was assessed in *Nostoc muscorum* (Galhano et al., 2011a,b). Exposure to the two contaminants resulted in a time and dose-dependent response of the cyanobacteria's antioxidant enzymes, although producing contrary effects: while molinate inhibited the organism's antioxidant enzymes' activity, bentazon exposure resulted in an increase of the same enzymatic set, apart from the enzyme GR, with decreased activity in response to increasing time and bentazon concentration. Bentazon reduces NADPH availability, which is needed by the enzyme for glutathione reduction, thus limiting the enzyme's action, which may explain the results obtained by the authors. Molinate is considered a more aggressive substance compared to other toxicants used for the same purpose, which is supported by the mentioned study – molinate may fully compromise the organism's antioxidant protection against ROS damage, while bentazon did not impair the organism's detoxification mechanism, which could still cope with bentazon contamination and prevent full damage.

The response of the freshwater microalgae *Scenedesmus obliquus* has been tested for numerous herbicides, such as atrazine, fenhexamid (Mofeed and Mosleh, 2013), lactofen and its metabolites desethyl lactofen and acifluorfen (Cheng et al., 2015) and flumioxazin (Geoffroy et al., 2004). Exposure to each of the six toxicants resulted, in general, in an increase of the algae's antioxidant enzymes' activity, indicating a successful activation of the cells' defense against ROS. However, after 24 h of flumioxazin exposure, most enzymes showed an activity decrease, indicating that the algae's antioxidant mechanism may effectively cope with a punctual contamination, but may not protect the organism from oxidative damage induced by a persisting contamination. This response was also observed in *Chlamydomonas reinhardtii*, regarding the microalgae's nitrate reductase (NR) activity, whose activity was only significantly reduced after 96 h of exposure (Fernández-Naveira et al., 2016). It is also worth noticing that *S. obliquus* SOD activity did not decrease at the highest lactofen concentration, but the enzyme's activity decreased with exposure to above mentioned lactofen metabolites. The higher sensitivity of the enzyme to the metabolites than the precursor substance - used as active agent of herbicides – raises the additional concern that, even if a pesticide degrades rapidly in the environment, which could normally be considered a favorable asset of the chemical, the resulting metabolites are not necessarily harmless. Metabolites may even have a greater impact than the original substance, inhibiting, at least, part of the antioxidant defense against ROS, as shown by Cheng et al. (2015). This potential scenario demands recognition and consideration especially during pesticides' design and definition of application practices. Moreover, different responses to pesticide mixtures need also to be considered. Mofeed and Mosleh, 2013 reported a "biphasic response" of *S. obliquus* antioxidant enzymes exposed to a mixture of atrazine and fenhexamid, revealing the real complexity of oxidative stress responses. In those conditions, enzymatic activity did not increase as significantly as registered in single-effect experiments, revealing an antagonistic interaction between the two chemicals.

Lemna minor is among the species of freshwater plant most used to assess responses to contaminants. Studies revealed that diuron, a phenylurea herbicide that blocks the interaction of plastoquinone with photosystem II, and the fungicide folpet increased the activity of enzymes GR, GST (Teisseire and Vernet, 2000, 2001) and APx (Teisseire and Vernet, 2001), while inhibiting CAT in a dose-dependent way (Teisseire and Vernet, 2000, 2001). Mitsou et al. (2006) conducted a study exposing *L. minor* to the herbicide Propanil, an anilide used to control barnyardgrass and other weeds in rice fields and other crops. Propanil has been reported to be harmful to aquatic species (Albanis et al., 1998; Pothuluri et al., 1991) and its metabolite 3,4-DCA is an endocrine disruptor (Crossland, 1990). In the referred study, the authors observed no alteration in the plant's antioxidant enzymes' activity in response to propanil exposure, however, there was an increase in 3,4-DCA in the medium. This suggested that the plant could cope with propanil contamination by metabolizing it through a detoxification path, which, in turn, may pose a challenge to organisms susceptible to 3,4-DCA action. In the presence of flumioxazin, *L. minor* antioxidant enzymes' activity increased after 24 h of exposure, persisting after 48 h, showing a potential for protection against oxidative stress in the event of toxicant persistence in the environment (Geoffroy et al., 2004).

Although herbicides may strike us as the most likely pesticide type to affect photosynthetic organisms, other classes also produce effects. The insecticide endosulfan, an OCP pesticide, is commonly used in crop fields due to its broad-spectrum and low cost, and has been reported to affect microbial populations, namely cyanobacteria (Satish and Tiwari, 2000). The response of *N. muscorum, Aulosira fertilissima* and *Anabaena variabilis* exposed to endosulfan was somewhat different depending on the species: *A. fertilissima* and *A. variabilis* showed an activity increase of

Table 1 (*continued*)

	Species	Pesticide	Concentration Range	Max. Exposure Period	Studied Enzyme	Lowest effect concentration	% Activity (in relation to control)	Reference
		Folpet (phthalimide – fungicide)			GR		≈+ 35 (24 h)	Teisseire and Vernet, 2001
					CAT		≈ + 15 (6 h)	
					GST		≈ + 38 (6 h)	
					APx		≈ + 50 (72 h)	
		Propanil (anilide – herbicide)	[0.01;0.5] x10^3 μg.l^{-1}	24 days	G-POD	*No significant changes found*		Mitsou et al., 2006
					GST	*No significant changes found*		
		Flumioxazin	3.6 μg.l^{-1}	48 h	CAT	3.6 μg.l^{-1}	+ 50% (24 h)	Geoffroy et al., 2004
					GR		~+ 40% (24 h)	
					APO		+ 28% (48 h)	
	Closterium ehrenbergii	Chlorine (Cl_2) (biocide disinfectant)	[1.4; 42.3] mM	72 h	SOD	28.21 mM	≈+200 (6 h)	Sathasivam et al., 2016
					CAT	1.41 mM	≈ 0 (72 h)	
					GSH	1.41 mM	≈ +260 (72 h)	
Triazine	*Chlamydomonas reinhardtii*	Atrazine (herbicide)	[10^{-4}; 2.00 × 10^{-3}] mM	96 h	NR	10^{-4}mM	− 40 (96 h)	Fernández-Naveira et al., 2016

antioxidant enzymes with higher concentration and exposure period to the toxicant, but only until the medium concentration used; *N. muscorum,* on the other hand, presented an increase in enzymatic activity with increasing substance concentration and exposure period until higher concentrations, suggesting a higher resistance of this species, among the three, to endosulfan exposure (Kumar et al., 2008).

The effect of OPP pesticides was assessed in the cyanobacteria *Chroococcus turgidus* (Kumar et al., 2014) and in the microalgae species *Chlorella pyrenoidosa* and *Merismopedia* sp. (Chen et al., 2016). Enzymes CAT and SOD of *C. turgidus* exposed to chlorpyrifos showed an increased activity after a two-day exposure to CP. Exposure to CP for 72 h and increasing concentration resulted in an increase of *Merismopedia* sp. SOD activity, while *C. pyrenoidosa* response consisted on the increase of the antioxidant enzymatic battery mainly at the lowest CP concentration. Regarding CAT activity stimulation, *C. pyrenoidosa* showed higher rises in the activity if CAT under the influence of the pollutant than *Merismopedia* spp., which may mean a higher sensitivity of *C. pyrenoidosa* to the presence of chlorpyrifos.

The green microalgae *Chlorella vulgaris* (Kurade et al., 2016) responded to exposure to diazinon with an increase of the algae's enzymatic antioxidant response up to 12 days of exposure.

Chlorine, Cl_2, is widely used as a disinfectant in water pools and reservoirs. The hypochlorite ion affects the metabolic, physiological and biological processes of the organisms, damaging cell membranes, proteins and nucleic acids. Exposure to Cl_2 induced oxidative stress in the microalgae *Closterium ehrenbergii* (Sathasivam et al., 2016), resulting also in a significant increase of SOD and GSH. It can be explained by the hypochlorous acid that is formed as a result of chlorination of proteins containing sulfhydryl groups, leading to the formation of disulfide bonds. GSH reduces the disulfide bonds to cysteines by serving as an electron donor. CAT did not present a significative increase, explained by the existence of some stressors that reduce the rate of protein turnover, also reducing CAT.

Table 1 provides more detailed information about the experimental results obtained in the studies regarding the response of photosynthetic organisms to pesticide exposure.

4.2. Zooplankton

Zooplanktonic species play a vital role in trophic webs as primary consumers. Hence, the assessment of impacts of contaminants on zooplankton species' biological processes and structure may provide early warning signs concerning potential harmful effects of the toxicants in whole trophic webs and ecosystems (Fossi et al., 2001). Although more studies concerning the response of zooplankton species to pesticide exposure have arose in the past years, further studies should be encouraged to evaluate the impacts in this group, crucial for trophic webs integrity and that may provide early warning signs of ecosystem

Table 2
Effects of pesticides in enzymatic activity of zooplankton species. When the value of the lowest concentration used in the mentioned studies is not specified in the article, the notation "*" is used in the table to express that value.

	Species	Pesticide	Concentration Range (mM)	Max. Exposure Period	Studied Enzyme	Lowest effect concentration	% Activity (in relation to control)	Reference
OPs	*Daphnia magna*	Triazophos (insecticide)	[0.05; 1.50]μg.l^{-1}	21 days	ChE (ATCh as substrate) ChE (BTCh as substrate)	1.50 μg.l^{-1}* 2.00 μg.l^{-1} * 0.50 μg.l^{-1}* 2.00 μg.l^{-1} *	+ 176.5 (10 days) ≈ − *55 (12 days)* + 174.2 (10 days) ≈ − 15 (21 days)	Li and Tan, 2011
		Chlorpyrifos (insecticide)	[0.01; 1.00] μg.l^{-1}		ChE (ATCh as substrate) ChE (BTCh as substrate)	0.10 μg.l^{-1} * 1.00 μg.l^{-1}* 0.10 μg.l^{-1} * 1.50 μg.l^{-1}*	+ 134.0 (8 days) ≈ − 50 (21 days) + 160.5 (8 days) ≈ − 40 (21 days)	
		Fenitrothion (insecticide)	[9.02 × 10^{-7}; 1.80 × 10^{-5}] mM	24 h	AChE CbE GST	9.02 × 10^{-7}mM	≈ − 32 ≈ − 50 ≈ − 22	Damásio et al., 2007
		Malathion (insecticide)	[1.00;16.00] μg.l^{-1}	96 h	AChE CbE GST	* * *	decreased decreased decreased	Trac et al., 2016
CMs		Carbaryl (insecticide)	1 × 10^{-4} mM	58 h	AChE	*	≈ − 80 (3 h) ≈ − 40 (20 h) ≈ − 80 (58 h)	Jeon et al., 2013
Diamids	*Daphnia magna*	Chlorantraniliprole (insecticide)	[1.00;4.00] μg.l^{-1}	21 days	SOD GPx CAT	4.07 μg.l^{-1} (24H), 2.11 μg.l^{-1} (48) 2.11 μg.l^{-1} (24H) 1.08 μg.l^{-1} (48)	− 68 (24H) −58 (48) −64 (24) − 132 (48)	Cui et al., 2017
		Cyantraniliprole (insecticide)	[3.00;12.00] μg.l^{-1}	21 days	SOD GPx CAT	6.21 μg.l^{-1} (24H) 11.48 μg.l^{-1} (48H) 6.21 μg.l^{-1} (24H) 11.48 μg.l^{-1} (24H) 6.21 μg.l^{-1} (48)	−86 (24H) −87 (48H) −75 (24H) * − 123 (48H)	
		Flubendiamide (insecticide)	[8.00;16.00] μg.l^{-1}	21 days	SOD GPx CAT	17.77 μg.l^{-1} (24H) 17.77 μg.l^{-1} (24H) *No significant change* 8.45 μg.l^{-1} (24H) 17.77 μg.l^{-1} (48)	−78 (24H) −60 (48H) −80 (24H) − 118 (48H)	
Neonicotinoids	*Daphnia magna*	Imidacloprid	[0; 0.625; 1.25; 2.5; 5; 10; 20; 40] mg.l^{-1}	21 days	AChE GST CAT	2.5 mg.l^{-1} 2.5 mg.l^{-1} 2.5 mg.l^{-1}	decreased decreased decreased	Jemec et al., 2007
		Guadipyr Cycloxaprid	[1.25;2.5;5.0] mg. l^{-1}	48 h	AChE CAT SOD AChE CAT SOD	0.84 mg.l^{-1} *No significant changes found* 3.59 mg l − 1 *No significant changes found*	decreased decreased	Qi et al., 2018

contamination.

Li and Tan (2011), observed the response of *Daphnia magna* cholinesterase enzymes to two OPPs: triazophos and chlorpyrifos. The authors reported that both compounds generate changes in the organisms' basal ChE activity at very low concentrations, showing hormetic responses during chronic exposure – at low concentrations both compounds induce an increase in the activity of ChE, but at higher concentrations they strongly inhibit the enzymes. Other OPP insecticides were used to evaluate the sensitivity and enzymatic responses of *D. magna* exposed to the toxicants, namely fenitrothion (Damásio et al., 2007) and malathion (Trac, Andersen and Palmqvist, 2016). Both studies reported a decrease in AChE activity with increasing concentrations of the pesticides, as expected, but this was also observed with other non-esterase enzymes, showing the broader and unpredictable response that pesticides cause on non-target organisms.

More recently, Cáceres et al. (2019) assessed the response of *Daphnia carinata* to toxicity of other two OPP insecticides, methyl parathion (MP) and its metabolite *p*-nitrophenol (PNP). After a 48 h exposure period, MP toxicity was observed at a much lower concentration than the needed for PNP toxicity to be detected, which only happened at concentration higher than the registered for the precursor compound. These results revealed that, in this case, the metabolite is significantly less toxic than the parent compound, allowing an effective antioxidant response of the organisms to the induced stress.

Contrarily to OPP pesticides, carbamates are reversible inhibitors of AChE, therefore being generally less toxic to organisms (Fukuto, 1990). The introduction of the CM carbaryl in the market was a breakthrough, as it is less persistent in the environment compared to OPP or OCl pesticides and said to rapidly detoxify by non-target organisms. Jeon et al. (2013) verified that *D. magna* AChE activity decreased abruptly within the first hours of carbaryl exposure, recovering, in fact, up to 60% of its activity after 20 h, probably due to AChE synthesis stimulation. This recovery was restricted, though, and AChE activity decreased once more as exposure to the pesticide continued. Nonetheless, organisms transferred to a pesticide-free medium recovered completely: daphnids immobilization, a consequence of AChE impairment, showed a practically linear relation to the exposure regime, while daphnids transferred to recovery regime, with no toxicant exposure, recovered from immobilization, in agreement with AChE activity recovery.

For the past decade, OPP and CM insecticides have been replaced with others of the diamid class, that present considerable efficiency and selectivity. Diamids act at organisms' muscle level, linking to receptors. For instance, the diamid insecticide chlorantraniliprole links to ryanodine receptors, leading to an excessive release of Ca^{2+} ions, resulting in muscle paralysis. Flubendiamide, causes disruption of Ca^{2+} balance, tampering with organisms' proper muscle function. The toxicity of three diamids – chlorantraniliprole, cyantraniliprole and flubendiamide – was assessed considering the antioxidant response of *Daphnia magna* to exposure to the three pesticides (Cui et al., 2017). The authors reported a significant decrease in SOD and GPx activities, resulting in an increase of ROS content. CAT activity significantly increased, which was explained by a compensation mechanism to remove H_2O_2 excess. These results were consistent with information reported elsewhere (Lavtižar et al., 2015).

Neonicotinoid insecticides, widely used in agricultural fields, are aimed at piercing-sucking animals, acting at nicotine receptors' level. Although being commercialized since the 1980′s, neonicotinoids have rapidly grown in markets and have more recently drawn public attention due to the growing resistance of target insects to the pesticide and the high toxicity they present to bees in particular (Simon-Delso et al., 2017). Studies over the years have reported the toxicity of neonicotinoids to non-target organisms, especially from aquatic environments. Imidacloprid (IMI), the first neonicotinoid to be commercialized, although presenting low acute toxicity to *Daphnia magna* (Sánchez-Bayo et al., 2016), has been reported to induce antioxidant stress to organisms while inhibiting their enzymatic antioxidant mechanisms (Jemec et al., 2007), posing a threat to the organisms' survival. The impact of IMI and the two newly developed neonicotinoids guadipyr (GUA) and cycloxaprid (CYC) was assessed by Qi et al. (2018) in *D. magna* exposed to different concentrations of the toxicants. The authors observed that exposure to GUA and CYC had no influence on antioxidant enzymes' activities, while IMI inhibited some antioxidant enzymes, thus inducing oxidative stress to the organisms and handicapping part of the response against the stress. Regarding AChE, the organism increased the enzyme's activity in response to IMI exposure, but AChE activity was inhibited by GUA and CYC even at the lowest concentrations. These findings demonstrate the different impacts that a same class of pesticides may have on a same non-target species, emphasizing the need to fully understand the exact toxicant in use, to minimize harmful, unpredicted effects to the environment.

Table 2 provides more detailed information about the experimental results mentioned regarding the use of enzymatic biomarkers to assess pesticide impact on zooplanktonic species.

4.3. Macroinvertebrates

Macroinvertebrates represent the trophic link between microorganisms/algae and fish/other vertebrates and are widely used as bioindicators due to their particular sensitivity to chemicals, large size (>50 μm) and relatively easy sampling. The group is also commonly used in laboratory toxicological studies to evaluate biochemical changes in response to contaminants, which may be used as biomarkers (Depledge and Fossi, 1994).

Kristoff et al. (2010) studied the effect of carbaryl in esterase enzymes (CbE and ChE) of the oligochaete *Lumbriculus variegatus* and the gastropod *Biomphalaria glabrata*. Both species presented inhibition of their esterase enzymes after exposure to the toxicant, as expected, but differed in some aspects: *L. variegatus* presented a more efficient recovery rate after transference to a toxicant-free medium compared to *B. glabrata*; it is also worth noticing that ChE was more sensitive than CbE to the action of the toxicant in the oligochaete, but the opposite was verified in the snail. As carbaryl is designed to target AChE, the results observed in the oligochaete were closer to the expected after carbaryl exposure. The contrasting results observed in the referred study confirm once more the unpredictability of toxicant effects, underlining the need for a cautious use of the substances and identification of the potentially affected non-target organisms.

The effect of the OPP pesticides parathion, its active metabolite paraoxon, known to be more aggressive than the precursor, and fenitrothion, designed to impact esterase enzymes, was also assessed in the freshwater clam *Corbicula fluminea* CbE and ChE (Basack et al., 1998). All chemicals inhibited the enzymes and paraoxon presented the highest toxicity, which is in agreement with the available literature.

Bakry et al. (2016) and Cacciatore et al. (2015) assessed the impact of OPP pesticides in the freshwater snails *Biomphalaria alexandrina* and *Planobarius corneus*, respectively, focusing on the response of antioxidants enzymes. Diazon and profenfos inhibited the response of the antioxidant enzymes of the snail, revealing a higher toxicity of profenfos (Bakry et al., 2016). Similar results had already been reported elsewhere (Youssef, 2010). Cacciatore et al. (2015) evaluated the response to both single and combined effects after exposure to commercial formulations of azinphos-methyl (AZM) and chlorpyrifos (PESTANAL®). In this case, antioxidant enzymes' activities were differently influenced. While some enzymes were not altered by either tested toxicants, others were significantly induced (e.g. GST and CAT) or inhibited (e.g. SOD).

The effect of chlorpyrifos was also assessed on the shrimp *Palaemonetes argentines* (Bertrand et al., 2016) and the snail *Lanistes carinatus* (Khalil, 2015). Both authors reported a significant decrease in the species' esterases, in particular mAChE, after exposure, while antioxidant enzymes' activity was induced. These findings are in accordance with other studies (Tyler Mehler et al., 2008) reporting an inhibited response of ChEs, especially AChEs, to OPP exposure. Indeed, CP produces an

Table 3
Effects of pesticides in enzymatic activity of macroinvertebrate species.

	Species	Pesticide	Concentration Range	Max. Exposure Period	Studied Enzyme	Lowest effect concentration	% Activity (in relation to control)	Reference
OPP	*Corbicula fluminea*	Chlorfenvinphos	[0.14 × 10^3;0.75 × 10^3] µg.l^{-1}	96 h	AChE	0.54 × 10^3 µg.l^{-1}	≈ − 45	Ramos et al.,2012
		Parathion (insecticide)	[20.00;80.00] µg.l^{-1}	24 h	CbE	6.87 × 10^{-5} mM	− 16	Basack et al., 1998
					ChE-S	1.37 × 10^{-4} mM	≈ − 25	
					ChE-P	6.87 × 10^{-5} mM	≈ − 25	
		Paraoxon (active metabolite of parathion)	[2.00;20.00] µg.l^{-1}		CbE	7.27 × 10^{-6} mM	− 26	
					ChE-S	7.27 × 10^{-6} mM	≈ − 23	
					ChE-P	1.82 × 10^{-5} mM	≈ − 21	
		Fenitrothion (insecticide)	[10.00;100.00] µg.l^{-1}		CbE	3.61 × 10^{-5} mM	− 15	
					ChE-S	1.80 × 10^{-4} mM	≈ − 20	
					ChE-P	1.80 × 10^{-4} mM	≈ − 25	
	Kiefferulus calligaster	Chlorpyrifos (insecticide)	[0.38;1.26] µg.l^{-1}	6 days	ChE	1.02 µg.l^{-1}	≈ − 30 (day 3)	Domingues et al., 2009
					GST	1.26 µg.l^{-1}	≈ − 40 (day 6)	
	Palaemonetes argentinus	Chlorpyrifos (insecticide)	[9.98 × 10^{-6}; 2.70 × 10^{-5}] mM	96 h	mAChE (in cephalotorax)	9.98 × 10^{-6} (mM)	− 40	Bertrand et al., 2016
					mAChE (in abdomen)	10.5 (ng.l^{-1})	− 55 (94.5 ng. l^{-1})	
					CAT (in cephalotorax)	8.99 × 10^{-5} mM	− 11.9	
					CAT (in abdomen)	31.5 ng.l^{-1}	− 11.9	
					cGST (in abdomen)	2.70 × 10^{-5}mM	*No changes*	
					GPx (in cephalotorax)	9.98 × 10^{-6} mM	− 3.10	
					GPx (in abdomen)	8.99 × 10^{-5} mM	− 2.60	
					SOD	*No changes*	*No change*	
	Chilina gibbosa	Azinphos-methyl	20 µg.l^{-1}	48 h	ChE	20 µg.l^{-1}	− 85	Cossi et al., 2015
					CbE		0	
	Biomphalaria alexandrina	Diazinon	[0.5–4] ppm	24 h	SOD	1.9 ppm	+23.53	Bakry et al., 2016
		Profenfos	[0.5–4] ppm		CAT	0.75 ppm	+19.69	
					GR		+50	
					LP		–23.69	
					TrxR		+54.17	
					SDH		+35.48	
					SOD		+48.53	
					CAT		+44.30	
					GR		+61.36	
					LP		−55.77	
					TrxR		+72.92	
					SDH		+72.90	
	Planobarius corneus	Azinphos-methyl	2.5 mg.l^{-1}	48 h	GSG	2.5 mg.l^{-1} 48 h	Decrease	Cacciatore et al., 2015
		Chlorpyrifos	7.5 µg.l^{-1}		GSSG	2.5 mg.l^{-1} 24 h	Decrease	
					SOD	2.5 mg.l^{-1}	Decrease	
					CAT	^{48}h2.5 mg.l^{-1} 48 h	decrease	
					GSG	7.5 µg.l^{-1} ^{24h}	decrease	
					GSSG	7.5 µg.l^{-1} 24 h	Decrease	
					SOD	$7^{.5}$ µg.l^{-1} ^{48}h	Decrease	
					CAT	$7^{.5}$ µg.l^{-1} ^{48}h	decrease	
CMs	*Biomphalaria glabrata*	Carbaryl	[0.05 × 10^3; 10 × 10^3] µg.l^{-1}	48 h	ChE	1 × 10^3 µg.l^{-1}	≈ − 26	Kristoff et al., 2010
	Lumbriculus variegatus		[0.006;5.00] µg.l^{-1}		Cbe	0.05 × 10^3µg.l^{-1}	≈ − 41	
					ChE	0.05 × 10^3 µg.l^{-1}	≈ − 36	
					CbE	0.5 × 10^3 µg.l^{-1}	≈ − 50	
					GST	2.62 × 10^{-2}mM	≈ + 45	
Pyrethroids	*Uniogibbus*	Cypermethrin (pyrethroidinsecticide)	[0.24; 0.36]mM	96 h	AChE	0.24 mM	− 38	Khazri et al., 2016
					SOD	0.36 mM	+ 63	
					CAT	0.24 mM	+ 67	
					GSH	0.36 mM	+ 65	
Triazine	*Channa Punctatus*	Atrazine	[4.238; 5.3000; 10.600] mg·l-1	15 days	SOD	4.238 mg·L-1	Increase	Nwani et al., 2010
					CAT	4.238 mg·L-1	Increase	
					GR	10.600 mg·L-1	Increase	

inactive phosphorylated enzyme out of AChE in its active site. There was also an increase of H_2O_2 levels that was accompanied by an enzymatic antioxidant response induction, in an attempt to reduce H_2O_2 to H_2O. Kahlil (2015) showed, moreover, that antioxidant enzymes' activity was eventually inhibited after 21 days of exposure. This could be either due to the inability of the organism to cope with long-term exposure to the toxicant, or to other compensatory mechanisms that would decrease the enzymes' activities. Exposure to fenitrothion in *Palaemonetes argentinus* (Lavarías and García, 2015), showed a similar response compared to the previous studies, with esterases inhibition in a time and dose-dependent

way and antioxidant enzymes' activity being induced.

The response to exposure to AZM of *Chilina gibbosa* was assessed through the analysis of cholinesterase enzymes and carboxylesterase activities by Cossi et al. (2015). After 46 h of exposure, expected results given the anti-esterase nature of OPPs were reported: ChE were more sensitive to the compound, being highly inhibited at lower concentrations, while CbE did not present activity change. The results were in accordance with a previous study (Bianco et al., 2013) where ChE showed inhibition at a concentration thousands of times lower than the needed to produce effects on CbE activity. However, some studies suggest that OPPs present a higher affinity to CbE than to ChE (Barata et al., 2004; Basack et al., 1998; Bianco et al., 2014; Kristoff et al., 2012; Ochoa et al., 2013; Ozretić and Krajnović-Ozretić, 1992), where CbE are more sensitive to AZM action, highlighting the unpredictability of the response of organisms' enzymes to a same contaminant, that may depend of various external factors.

Concerning pyrethroid pesticides, Khazri et al. (2016) tested the insecticide cypermethrin on *Unio gibbus*, reporting a decrease in AChE activity, a significant increase in SOD and CAT activity, and decrease of GSH. Pyrethroids can modulate the quantity of the neurotransmitter acetylcholine, thus decreasing the need for AChE, while inducing oxidative stress, stimulating the antioxidant mechanisms of the cells.

Table 3 provides more detailed information about the experimental results obtained in the studies above mentioned regarding the response of macroinvertebrates to contaminant exposure.

4.4. Fish

Fish present a diverse group of animals occupying various positions in trophic webs, from being in direct higher position to zooplankton, to equal or higher position to macroinvertebrates. Fish are commonly the direct food source of various aquatic and non-aquatic animal communities, including humans, thus being a more relatable to human physiology kind of bioindicator. As bioindicators, fish are generally more resistant to higher concentrations of toxicants, mainly due to their size and physiological and metabolic filtering mechanisms, bioaccumulating all sorts of substances, as well as respond to low concentration of mutagenic substances (Çavaş and Ergene-Gözükara, 2005).

The commercial formulation Clorofox®, whose active agent is clorpyrifos, is used widely used in a South American region named Pampean, to control various insects and crops (Brodeur et al., 2011). Studies conducted over the years (e.g. Botté et al., 2012; De Silva and Samayawardhena, 2005; Ozcan Oruç, 2010) have reported the harmful effects of this pesticide in fish, namely behavioral, neurological and reproductive. Bonifacio et al. (2017) studied the response of two fish species to exposure to the pesticide: the ten spotted live-bearer (*Cnesterodon decemmaculatus*), a species tolerant to stressful conditions and commonly used as model for ecotoxicological assays, and the Uruguay tetra (*Cheirodon interruptus*), which is, in the other hand, a sensitive species to such conditions and rarely used in toxicological tests (Ossana et al., 2016; Vera-Candioti et al., 2014; Campana et al., 1999). Responses to the commercial formulation was assessed through analysis of the enzymatic activity of AChE, CAT, GST, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) in the above mentioned fish species exposed to different concentrations of the toxicant for 48 h. AChE activity measured in fish brain and muscle did not register differences between exposure concentrations in *C. interruptus*, but decreased significantly in *C. decemmaculatus* in a dose-dependent way. However, as *C. interruptus* is known to be more sensitive to changes in the environment, the experimental exposure concentrations were one order of magnitude lower compared to the ones used for *C. decemmaculatus* – the lower experimental concentration of *C. decemmaculatus* corresponded to the higher exposure concentration used for *C. interruptus*. CAT activity was assessed in gills, liver, brain and muscle tissue of both species but was not detected in either brain or muscle of the individuals. A significant decrease in CAT activity was only reported in *C. decemmaculatus* liver at the highest exposure concentration, showing no differences in the species gills. The enzymatic activity of GST did not vary significantly in liver or gills of *C. decemmaculatus*, and was not present in brain samples, but decreased in muscle tissue at the lowest substance exposure. Concerning *C. interruptus*, the enzyme's activity did not present changes in the brain, liver or gills of the species, and was not detected in muscle samples.

Narra et al. (2017) assessed the response of *Clarias bathrachus* exposed to CP, as well, and monocrotophos (MCP). Hemalatha et al. (2016) also tested the response to an OPP pesticide – quinalphos – in the common carp *Cyprinus carpio*. In both cases, specimens were subjected to longer periods of exposure to the contaminants (days) and Narra et al. (2017) also subjected organisms to a subsequent depuration period, to assess fish ability to detoxify from the contaminants. In both studies, the antioxidant enzymatic defenses in fish livers were activated, with SOD, CAT, LPO, GST and GSH increasing with exposure period, reflecting the organisms' balancing mechanism against abnormal formation in ROS. Narra et al. (2017) reported, however, an inhibition of GPx, and of brain and muscle AChE and gills ATPase in response to pollutant exposure. Nonetheless, levels of all enzymes began to be restored with depuration, being almost completely restored after 30 days. The results obtained provided not only confirmation of the harmful effects of the toxicants in fish species, but also further evidence of the potential effectiveness of depuration processes in fish decontamination after exposure to pollutants, namely pesticides.

Clasen et al. (2018) studied the effect of five commonly used pesticide formulations in rice fields in the common carp, which inhabit those same fields and provide protection against rice crops' pests and serve as additional income for rice producers. The authors assessed the impact of the insecticides lambda-cyhalothrin + thiamethoxam (Endigo® ZC) and clorantraniliprole and of the fungicide tebuconazole + trifloxystrobin in fish exposed to the toxicants for 100 days. The authors reported that all pesticides caused oxidative stress with adverse consequences in the organisms, which responded to contamination by increasing the activity of antioxidant enzymes CAT and GST, in a protection effort against ROS, at the same time that lipid peroxidation and protein oxidation increased in fish liver and muscle. AChE levels were also altered in response to toxicant exposure, with lowered activity in the brain, compared to control organisms. Moreover, lambda-cyhalothrin and tebuconazole bioaccumulated in carp muscles, rising the concern of potential health risks to humans associated with the consumption of fish grown in integrated systems which are still treated with aggressive pesticides, that eventually bioaccumulate in fish to be commercialized.

Also regarding OPP pesticides, Guerreño et al. (2016) subjected the fish species *Odentesthes hatcheri,* an Argentinian autochthonous species with commercial interest and ecological relevance, and *Jenynsia multidentata*, another model species in toxicological studies in South America to AZM. The authors reported a significant inhibition of brain AChE activity in both species, while presenting a dual response, depending on the contaminant concentration, in *O. hatcheri* muscle and no change in *J. multidentate* muscle. CbEs activity response to contaminant exposure showed a different pattern, with no changes in *O. hatcheri* brain but increasing significantly in *J. multidentata*, as well as in *O. hatcheri* gills and both species' muscle tissue. The results confirm the expected, since organophosphates like azinphos-methyl inhibit acethylcholinesterase, while carboxylesterases are the mains OPPs detoxification enzymes, hence CbE activity was expected in order to protect organisms from AChE inhibition (Fukuto, 1990; Jokanović, 2001). Antioxidant enzymes were also assessed by Guerreño et al. (2016), reporting inhibition of GR and CAT activities and a slight increase of GST activity in *O. hatcheri*, while no significant changes were found in *J. multidentata* antioxidant enzymes. In general, *O. hatcheri* showed a higher sensitivity to AZM.

Propiconazole (PCZ) is used against fungi responsible for several diseases in humans and other organisms. (Tabassum et al., 2016) exposed *Channa punctata* (bloch fish), a common species in Asia, different sub-lethal concentrations of PCZ for 96 h period, after which

Table 4
Effects of pesticides in enzymatic activity of fish species. When the value of the lowest concentration used in the mentioned studies is not specified in the article, the notation "*" is used in the table to express that value.

	Species	Pesticide	Concentration Range	Max. Exposure Period	Studied Enzyme	Lowest effect concentration	% Activity (in relation to control)	References
	Cnesterodon decemmaculatus *Cheirodon interruptus*	Chlorpyrifos	[0.084; 0.84] $\mu l.l^{-1}$ [0.84;8.4] $\mu l.l^{-1}$	48 h	AChE CAT GST AChE CAT GST	0.84 $\mu l.l^{-1}$ 0.84 $\mu l.l^{-1}$ *No significant changes found* *No significant changes found* 0.84 $\mu l.l^{-1}$ *No significant changes found*	increase (muscle) Decrease Decrease (brain) 	Bonifacio et al., 2017
	Clarias bathrachus	Chlorpyrifos Monocrotophos	1.65 mg. l^{-1} 2.14 mg. l^{-1}	15 days	SOD CAT LPO GPx GST GSH SOD CAT LPO GPx GST GSH	1.65 $mg.l^{-1}$ (30 d) 1.65 $mg.l^{-1}$ (30 d) 1.65 $mg.l^{-1}$ (30 d) 1.65 $mg.l^{-1}$ (30 d) 1.65 $mg.l^{-1}$ (30 d) 1.65 $mg.l^{-1}$ (30 d) 2.14 $mg.l^{-1}$ (15 d) 2.14 $mg.l^{-1}$ (15 d) 2.14 $mg.l^{-1}$ (15 d) 2.14 $mg.l^{-1}$ (15 d) 2.14 $mg.l^{-1}$ (15 d) 2.14 $mg.l^{-1}$ (15 d)	Increase Increase Increase Decrease Increase Increase Increase Increase Increase Decrease Increase Increase	Narra et al., 2017
OPs	*Cyprinu scarpio*	Diazinon (insecticide)	[1.18×10^{-8}; 5.91×10^{-8}]mM	30 days	AChE (in gills) AChE (in muscle) Na^+K^+ ATPase (in gills) Na^+K^+ ATPase (in muscle) Na^+K^+ ATPase (in kidney) SOD (in gills) SOD (in muscle) SOD (in kidney) CAT (in gills) CAT (in muscle)	1.18×10^{-8} mM 1.18×10^{-8} mM 1.18×10^{-8} mM 5.91×10^{-8} mM 5.91×10^{-8} mM 1.18×10^{-8} mM 5.91×10^{-8} mM 1.18×10^{-8} mM 5.91×10^{-8} mM 1.18×10^{-8} mM	− 32.5 (30 days) ≈ − 50 (30 days) + 72.2 (5 days) ≈ − 15 (30 days) ≈ − 30 (30 days) + 102.17 (5 days) ≈ + 40 (30 days) + 46.19 (5 days) + 51,09 (5 days) ≈ − 20 (30 days)	Oruç and Usta, 2007
	Cyprinus carpio	Quinalphos (insecticide)	[1.09; 11.00] $\mu l.l^{-1}$	20 days	SOD (in liver) CAT (in liver) GST (in liver)	1.09 $\mu l.l^{-1}$ (5 days) 1.09 $\mu l.l^{-1}$ (5 days) 1.09 $\mu l.l^{-1}$ (5 days)	Significant increase Significant increase Significant increase	Hemalatha et al., 2016
	Odentesthes hatcheri *Jenynsia multidentata*	Azinphos-methyl	[3.15×10^{-4}; 3.15×10^{-2}]mM [1.58×10^{-2}; 1.58×10^{-1}]mM	96 h	AChE (in brain) CarbE (in brain) AChE (in muscle) CarbE (in muscle) GR (in gills) GST (in gills) CAT (in gills) CarbE (in gills) AChE (in	0.5 $\mu g.l^{-1}$ 0.1 $\mu g.l^{-1}$ 0.1 $\mu g.l^{-1}$ 0.1 $\mu g.l^{-1}$ 0.1 $\mu g.l^{-1}$ 10 $\mu g.l^{-1}$ 10 $\mu g.l^{-1}$ 10 $\mu g.l^{-1}$	− 50 0 − 14 + 50 − 30 ≈+ 2.7 − 46 ≈-43 −42 + 33 0 + 25 0 0	Guerreño et al., 2016

(*continued on next page*)

Table 4 (*continued*)

	Species	Pesticide	Concentration Range	Max. Exposure Period	Studied Enzyme	Lowest effect concentration	% Activity (in relation to control)	References
					brain)		0	
					CarbE (in brain)		0	
					AChE (in muscle)			
					CarbE (in muscle)			
					GR (in gills)			
					GST (in gills)			
					CAT (in gills)			
					CarbE (in gills)			
Others	*Cyprinus carpio*	lambda-cyhalothrin + thiamethoxam (Endigo ®) (insecticide)	0.2 $l.ha^{-1}$	100 days	CAT	0.2 $l.ha^{-1}$	Increase (liver)	Clasen et al., 2018
					GST	0.2 $l.ha^{-1}$	Increase (brain, live rand gills)	
					AChE	0.2 $l.ha^{-1}$	Decrease (brain)	
		Clorantraniliprole (insecticide)	50 $g.ha^{-1}$		CAT	50 $g.ha^{-1}$	Increase (liver)	
					GST	50 $g.ha^{-1}$	Increase (brain, liver and gills)	
					AChE	50 $g.ha^{-1}$	Decrease (brain)	
		tebuconazole + trifloxystrobin (Nativo ®) (fungicide)	0.75 $l.ha^{-1}$		CAT	0.75 $l.ha^{-1}$	Increase (liver)	
					GST	0.75 $l.ha^{-1}$	Increase (brain, live rand gills)	
					AChE	0.75 $l.ha^{-1}$	Decrease (brain)	
	Channa punctata	Propiconazole (fungicide)	[1.46; 14.61]mM	96 h	GST (in liver)	*	−49 (10.28 μM)	Tabassum et al., 2016
					GST (in kidney)		− 55 (8.11 μM)	
					GST (in gills)		− 38 (2.36 μM)	
					GPx (in liver)		−43 (9.81 μM)	
					GPx (in kidney)		−55 (5.47 μM)	
					GPx (in gills)		− 57(6.97 μM)	
					CAT (in liver)		− 46 (24.43 μM)	
					CAT (in kidney)		− 59 (11.21 μM)	
					CAT (in gills)		− 48 (27.45 μM)	

specimens' kidney, liver and gills were analyzed for GST, GPx and CAT activities. The authors reported a significant decrease in the enzymes' activity in relation to the control group, namely of 46%, 59% and 48% in liver, kidney and gills respectively, thus showing an imbalanced pro-oxidant and antioxidant defense mechanism due to diminished antioxidant content or excessive ROS production.

The effect of the herbicide Rasayanzine, a commercial formulation of atrazine, one of the most common pesticides used in agriculture, was assessed on the spotted snakehead, by Nwani et al. (2010). Atrazine is designed to inhibit photosynthesis in plants and was thus not thought to affect animals. However, studies have shown that it affects animals in various ways (de Campos Ventura et al., 2008; Weigand et al., 2001). The effect of the herbicide on the tested fish was assessed based on, among other indicators, SOD, CAT and GR activities in fish liver. SOD and CAT activities significantly increased in a concentration and time-dependent pattern, but CAT activity began to decrease after half of the exposure period, after which it continuously decreased until the end of the exposure period. GR, on the other hand, showed a significant increase only by the end of the exposure period, showing a completely different pattern than the remaining enzymes.

Copper sulphate ($CuSO_4$) is a widely used algaecide and fungicide in aquaculture and agriculture productions (Chen and Lin, 2001; Lasiene et al., 2016). Kirici et al. (2017) studied the effect of $CuSO_4$ in the freshwater fish *Capoeta umbla*, commonly known as the Tigris scraper, as an inducer of oxidative stress in the fish liver, kidney and gills. The activity of the antioxidant enzymes G6PD, GR, SOD, CAT and GPx, was assessed in the tissues referred to evaluate the substance effect after a 96 h exposure to a range of contaminant concentrations. The experiment resulted in a dose-dependent significant decrease of GR, G6PD and GPx activity and CAT and SOD activity increase in all tissues, regardless time of exposure.

Similarly to other groups, most studies addressing the impact of pesticides in fish species are of single pesticide exposure. However, the study of combined effects of pesticides is closer to real situations, as there is a higher probability of aquatic systems to be contaminated by more than one pesticide. Ballesteros et al. (2017) aimed to assess the biomarker response of the fish species *Jenynsia multidentata* in a river subjected to anthropic pressures, namely of pesticide introduction. Specimens in cages were placed in four sites of the river with different contamination conditions. Enzymatic responses to contaminants were assessed in gills, liver, brain and muscle. The authors observed differences in the activity of the enzymes GST, CAT, AChE and BChE responding to a combination of different stress factors (for example the combination of xenobiotics' presence and the hydrological conditions of the study sites), which allowed a separation of the sites according to the multi-factor set of conditions that influenced each site. Although the need for further studies to determine the actual pesticides present in the basin is referred, the results obtained are consistent with other studies assessing the impact of the pesticides endosulfan, glyphosate, atrazine and chlorpyrifos regarding GST and CAT. The authors also reported the presence of high levels of BChE in fish tissues and, knowing the presence of OPPs in the systems, it was concluded that BChE may be playing a detoxification role in fish; the levels of AChE found were related with the presence of heavy metals (as copper, cadmium and aluminum) in the system, and are consistent with findings of similar studies.

Table 4 provides further detailed information regarding the experimental results above mentioned and described, concerning the response of fish species to pesticide exposure.

5. Conclusions and future perspectives

The present review reflects on the wide use of enzymatic biomarkers, namely of antioxidant enzymes and esterases, the later to a lesser extent, to assess and characterize the impact of pesticides in various freshwater organisms' groups. These biomarkers have proven over the years to be an effective and resourceful means to address the issue, however, there is also the need to highlight some constraints of the use of enzymes. As shown throughout the present manuscript, enzymatic responses to contaminants are not necessarily always linear or constant even in response to a same pesticide or class of pesticides. Several factors need to be taken into account, and using a set of biomarkers to determine a general response of a certain species or group to specific pesticides could be a more effective strategy to adopt. There is the need to effectively consider the results obtained by the numerous studies here present, the recommendations they provide concerning the harmful impacts of pesticide use in non-target communities, as well as the critical assessment of the imperative need to better control and regulate pesticide design and application. Reports on the severe harmful effects of various pesticides in different organisms, across trophic webs, provide strong support to encourage the urgency of the production of pest-control substances more species-specific, less toxic to non-target species and less persistent in the environment. Lowering pesticides ecological impact and strengthening the regulation for pesticide use is crucial to preserve ecosystems and prevent unknown effects on non-target organisms, still yet to be discovered and with potential irremediable consequences.

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.

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