



# Oxidative stress in the freshwater shrimp *Caridina africana* following exposure to atrazine

Gregg J van Rensburg<sup>1</sup> · Victor Wepener<sup>2</sup> · Suranie Horn<sup>2,3</sup> · Richard Greenfield<sup>1</sup>

Received: 13 January 2022 / Accepted: 5 April 2022 / Published online: 27 April 2022  
© The Author(s), under exclusive licence to Springer Science+Business Media, LLC, part of Springer Nature 2022

## Abstract

The toxicity of pesticides to non-target organisms continues to be important in understanding the dynamic interactions between anthropogenic chemicals and ecosystem health. This study assesses biochemical markers to determine the effects that varying concentrations of atrazine (13.1–5557 µg/l) have on the freshwater shrimp, *Caridina africana*. Exposure and oxidative stress biomarkers were analysed and followed by univariate, integrated biomarker response v2 (IBRv2) and Kendall Tau correlation statistical analyses, to gain insight into the concentration-dependent responses. Oxidative stress biomarkers such as reduced glutathione content (GSH), glutathione-S-transferase activity (GST), superoxide dismutase activity (SOD) and catalase activity (CAT) were significantly correlated with increasing atrazine exposure concentration ( $p < 0.01$ ). Bimodality has been seen when looking at both the univariate statistically significant differences as well as the IBRv2, with the first peak at 106.8 µg/l and the second peak at 5557 µg/l atrazine. The results indicate that while individual responses may indicate statistically significant differences, using correlation and integrated statistical analysis can shed light on trends in the adaptive response of these.

**Keywords** Atrazine · Biomarkers · Decapod · Integrated biomarker response v2

## Introduction

Global pesticide usage in 2012 was over  $2.64 \times 10^9$  kg of applied active ingredient with projections estimating an increase up to  $3.47 \times 10^9$  kg in 2020 (Atwood and Paisley-Jones 2017; Zhang 2018). Herbicides account for up to 49% of global pesticide consumption (Atwood and Paisley-Jones 2017; De et al. 2014). Atrazine, one of the most commonly used herbicides, is predominantly a broad-leaf, pre and post emergence, herbicide applied to corn, sorghum, sugarcane and many other crop types (Griboff et

al. 2014). Contamination of surface waters with atrazine occurs through various routes such as aerial drift, run-off and seepage and has been detected in rain, fog, seawater and arctic ice (Du Preez et al. 2005; Jablonowski et al. 2011). Although there is still debate over the endocrine-disrupting properties of atrazine, it has been banned in the European Union due to environmental concentrations exceeding permissible drinking water concentration limits of 0.1 µg/l (Sass and Colangelo 2006). The ban was a proactive precautionary measure, however in many countries agricultural use is ongoing. Atrazine is persistent in water and soils and has been detected in wells in Canada and the USA at 74 µg/l and 140 µg/l respectively (Graymore et al. 2001). Examples of guideline maximum concentration considered safe for drinking water are 5 µg/l for Canada, 3 µg/l for the USA and 2 µg/l for South Africa (Canada Health 1993; DWAF 1996; USEPA 2007).

South Africa has banned industrial applications of atrazine, but the herbicide is still permitted for use in agriculture. Maize is the most cultivated crop in South Africa and in 2009 maize accounted for 87.83% of national atrazine usage which equated to  $8.9 \times 10^5$  kg with national usage of approximately  $1.01 \times 10^6$  kg (Dabrowski 2015). Detected

✉ Richard Greenfield  
rgreenfield@uj.ac.za

<sup>1</sup> Department of Zoology, Kingsway Campus, University of Johannesburg, PO Box 524, 2006 Auckland Park, South Africa

<sup>2</sup> Water Research Group, Unit for Environmental Science and Management, North-West University, Potchefstroom, South Africa

<sup>3</sup> Environment & Health Research Unit, South African Medical Research Council (SAMRC), 7505 Tygerberg, South Africa

atrazine concentrations in heavy maize producing areas of South Africa in the highveld region, vary significantly ranging between  $<0.005$   $\mu\text{g/l}$  to  $14.97$   $\mu\text{g/l}$  (Ansara-Ross et al. 2012; Dabrowski 2015; Rimayi et al. 2018), though some studies have reported surface water atrazine contamination up to  $53\,000$   $\mu\text{g/l}$  in streams adjacent to agricultural areas (Davies et al. 1994). The biochemical impact on non-target aquatic organisms needs to be assessed to determine the impact of potential exposure.

The family Atyidae accounts for more than 58% of all freshwater shrimp with *Caridina* being the largest genus, accounting for 290 of the 443 recognised species in the family. Nearly one-third of freshwater shrimp species are classified as threatened or near threatened, of which over 68% are primarily impacted by urban and agricultural runoff (De Grave et al. 2015; de Mazancourt et al. 2019). The distribution and sensitivity to environmental contamination make *Caridina sp.* appropriate bioindicators. In a field study we recently showed that biomarker responses in *Caridina nilotica* were able to reflect organic pollutant exposure (Jansen van Rensburg et al., 2020). Therefore, *Caridina sp.* were chosen for this study and were identified as *Caridina africana* using the key of Richard and Clark (1995).

The aim of this paper was to determine the oxidative stress incurred at different atrazine concentrations in *C. africana* under short term testing conditions. This was achieved using biomarkers of exposure and oxidative stress namely acetylcholinesterase activity (AChE), reduced glutathione (GSH), glutathione S-transferase (GST), superoxide dismutase (SOD), catalase activity (CAT) and malondialdehyde content (MDA). An integrated biomarker response (IBR) was performed to assess the combined effect at each exposure concentration. The IBRv2 allows for a standardized approach to interpreting the effects that toxicants have on an organism's body. As the organisms have to cope with greater ATZ concentrations, it is hypothesised that increasing exposure concentrations will result in increased expression of the selected biomarkers, which will be reflected in the correlations and IBRv2.

## Materials and Methods

### Sampling and experimental design

Ethics clearance for the study was granted by the University of Johannesburg's Ethics committee (Ref# 2016-09-23/Greenfield\_Jansen van Rensburg). *Caridina africana* were collected from the Vaal River system (Standerton, Mpumalanga Province, South Africa) using a one-millimetre mesh size net and transported to the Department of Zoology Aquarium at the University of Johannesburg. The organisms were

acclimated under laboratory conditions for five months. The setup for the exposure is based on the USEPA's 96 h acute toxicity testing for fish (Muller et al. 2011; USEPA 2002), while being cognisant of a pseudoreplicated experimental design. The methodology considers each individual organism as the experimental unit because the dependent variables are measured at the sub-organism level (Schank and Koehnle 2009; Tincani et al. 2017). Five test organisms, in duplicate per concentration, were placed in 500 ml beakers in matured borehole water (pH  $-7.3$ ; electrical conductivity  $-110$   $\mu\text{S/cm}$ ; temperature  $-21.8$   $^{\circ}\text{C}$ ) under constant aeration. Results from each organism were combined per exposure concentration for statistical analysis. The organisms were acclimated in the beakers for 24 h and were not fed for the duration of the acclimation and exposure test. Atrazine (CAS #1912-24-9, purity 99%, Sigma-Aldrich) stock was made using ultrapure water at a concentration of  $20$   $\text{mg/l}$  and was used for all the exposure concentrations. The nominal concentrations of atrazine used in the 96 h exposures were 0, 10, 25, 63, 156, 391, 977, 2441 and  $6104$   $\mu\text{g/l}$ . The selected concentrations were chosen to cover a large range and were calculated as a ratio of 1:2.5 starting at  $10$   $\mu\text{g/l}$ , slightly higher than suggested by the OECD (2019). Water samples were taken at the beginning of the exposure period in glass bottles, to confirm accurate exposure concentrations and were representative of both duplicate beakers. Following the exposure period organisms were collected in cryotubes and euthanised by flash freezing in liquid nitrogen and transferred to a  $-80$   $^{\circ}\text{C}$  freezer until analysis.

### Atrazine Extraction and Analysis

Water samples were prepared for gas chromatography – micro electron capture detector (GC- $\mu\text{ECD}$ ) analysis using a modified dispersive liquid-liquid microextraction method of Della-Flora et al. (2018). Briefly,  $2.5$  g NaCl was dissolved in  $25$  ml water samples before the addition of  $3\,565$   $\mu\text{l}$  dispersive/extraction solvent. The mixture was made by mixing HPLC grade dichloromethane (DCM,  $\geq 99.8\%$ ) with acetone ( $\geq 99.8\%$ ) at a ratio of 550:163. The mixture was rapidly vortexed and centrifuged at  $1\,000 \times g$  for 5 min. An aliquot of  $400$   $\mu\text{l}$  of organic phase was transferred to a GC vial where it was evaporated under a light stream of nitrogen before being reconstituted in  $100$   $\mu\text{l}$  ethyl acetate and analysed. The methodology followed the USEPA method 508.1, version 2 (Munch 1995), with slight adjustments. A Hewlett Packard 6890 GC- $\mu\text{ECD}$  fitted with an Agilent DB-5ms UI fused silica capillary column ( $30$  m  $\times$   $0.25$  mm  $\times$   $0.25$   $\mu\text{m}$  film thickness) was used for the separation and detection of atrazine. Helium was used as the carrier gas, with the total flow rate at  $1.5$  ml/min, including nitrogen as the make-up gas, set to  $45$  ml/min. Splitless injection of a

10 µl sample was performed with an inlet temperature set at 250 °C. The GC oven temperature program was as follows: initial temperature 60 °C and 1 min hold; ramp at 20 °C/min to 160 °C and hold 3 min; ramp 3 °C/min to 275 °C with no hold; and finally, 2 °C/min to 310 °C with no hold. The detector temperature was set at 320 °C.

The method was validated for linearity, precision and accuracy. Linearity of the calibration curve was assessed by the coefficient of determination ( $R^2$ ) as close to 1.0 as possible (Miller and Miller 2010). The standards were analysed in triplicate and were injected in order of increasing concentration with blank injections in between to prevent carryover. Standard concentrations were prepared to deliver a linear range and resulted in an  $R^2$  of 0.997. Precision was expressed as relative standard deviation (%RSD) by injecting 6 replicates of spiked blank samples (spiked with 350 µg/l) and resulted in 12%RSD. Accuracy for the spiked water samples was between 75 and 130%.

### Biomarker Analysis

Frozen organisms were thawed and whole organisms were accurately weighed ( $\pm 0.001$  g) and placed in individual microcentrifuge tubes. Individuals were homogenized on ice in one millilitre homogenizing buffer (50 mM potassium phosphate buffer, pH 7.4 with 0.9% NaCl and 1 mM EDTA) and centrifuged for 25 min at 25 000  $\times$  g. The resulting supernatant was transferred to new tubes and was used for all biomarker protocols which were all tested in triplicate with the homogenizing buffer as a blank. All biomarkers were read in 96-well microtiter plates and analysed on an ELx 800 (colorimetric) and FLx 800 (fluorometric) universal microplate reader (Biotek Instrument Corp).

Acetylcholinesterase activity was determined according to Ellman et al. (1961). A reaction mixture of 90 mM potassium phosphate buffer (pH 7.4), 30 mM acetylcholine iodide and 10 mM DTNB was added to a microplate. The plate was incubated at 37 °C for 5 min after which 5 µl of sample supernatant was added to start the reaction. The reaction was read kinetically at 405 nm for 6 min with 1 min intervals. Reduced glutathione content was measured following the method of Cohn and Lyle (1966). Two hundred and fifty microliters of sample supernatant were transferred to a microcentrifuge tube to which 25%  $H_3PO_4$  was added and the tubes were placed on ice for 10 min. The samples were then centrifuged at 5 000  $\times$  g for 10 min at 4 °C. One hundred microliters of supernatant was transferred to a new tube and diluted with 1 ml of distilled water before being added to the microplate with 100 mM sodium phosphate buffer (pH 8.0) and 0.1% o-phthalaldehyde. The plate was incubated at room temperature for 15 min in the dark before fluorescence was measured at 350 nm excitation and

420 nm emission. Glutathione S-transferase was determined according to Habig et al. (1974). To a microplate, 270 µl of reaction mixture containing potassium phosphate buffer (0.1 M, pH 6.5) and 1 mM GSH was added to 10 µl of sample supernatant. The reaction was initiated with 20 µl of 1 mM CDNB and absorbance read at 25 °C every 30 s for 3 min at 340 nm. Superoxide dismutase was read following the protocol of Del Maestro and McDonald (1985). The reaction mixture contained 50 mM Tris DTPA, 24 mM pyrogallol and 5 µl of sample supernatant. The mixture was immediately read kinetically in the dark at 420 nm over a 5 min period with 30 s intervals. Catalase activity was determined following the protocol of Cohen et al. (1970). Ten microliters of sample were loaded into the microplate, to which 100 µl of cold 20 mM  $H_2O_2$  was added. The mixture reacted for 3 min and was stopped by the addition of 20 µl  $H_2SO_4$  (6 N). Potassium permanganate (140 µl, 2 mM) was added and the plate was immediately read at 490 nm. Malondialdehyde content was measured following the protocol of Ohkawa et al. (1979). To a microcentrifuge tube 20 µl of sample, 50 µl SDS, 375 µl acetic acid, 375 µl thiobarbituric acid and 175 µl of distilled water were added and placed in a 95 °C water bath for 30 min. After cooling 250 µl distilled water and 1250 µl butanol:pyridine (15:1, v:v) were added and after centrifugation the supernatant was read at 532 nm. All biomarkers were expressed per milligram protein (Bradford 1976), to allow for standardization.

### Statistical Analysis

Atrazine concentrations determined through GC- $\mu$ ECD analysis were used as the independent variables in the statistical analysis and graphing thereof. Univariate biomarker data are presented as box and whisker plots with the Kendal Tau's correlation coefficient ( $\tau$ ) and statistical significance ( $p < 0.05$  or  $p < 0.01$ ) indicated. Box and whisker plots, per exposure concentration, have been generated from all of the individual organism's biomarker response data. Kendal Tau's correlation analysis was used to identify overall trends in the data with concentration group as the independent variable. It should be borne in mind that  $\tau$  interpretation is not analogous to spearman correlation coefficient as  $\tau$  will underestimate by between 66 and 75% (Strahan 1982). Certain raw data failed assumptions of parametric tests and non-parametric equivalents were selected. To test for significance between group means, the Kruskal-Wallis test (H test statistic) was used, and through a Bonferroni correction the  $\alpha$  threshold was set at 0.0063 for pairwise comparisons. Eight comparisons were made between the control group and each exposure group, as these were of primary interest. The integrated biomarker response metric was calculated according to Beliaeff and Burgeot (2002) and modified by

Sanchez et al. (2013). The modification introduced the concept of reference deviation and is aptly named integrated biomarker response version 2 (IBRv2). Briefly, for each biomarker, a log transformation is applied to the ratio between individual response data ( $X_i$ ) and the mean of the reference data ( $X_0$ ):  $y_i = \log(x_i/x_0)$ .

$Y_i$  is then standardized after calculating the general mean ( $\mu$ ) and standard deviation ( $\sigma$ ):  $z_i = (y_i - \mu) / \sigma$ . The reference deviation ( $A_i$ ) was used to create radar plots and is calculated as the difference between the mean of the standardized response ( $Z_i$ ) and the mean of the reference or control response data ( $Z_0$ ):  $A_i = z_i - z_0$ .

Finally, the IBRv2 is calculated as the sum of the absolute values of the  $A$  responses:  $IBRv2 = \sum |A_i|$

Statistical tests were run on IBM® SPSS® Statistics v27 with graphical representation generated on GraphPad Prism v8. Microsoft Excel was used to calculate and generate the radar plots for the IBRv2.

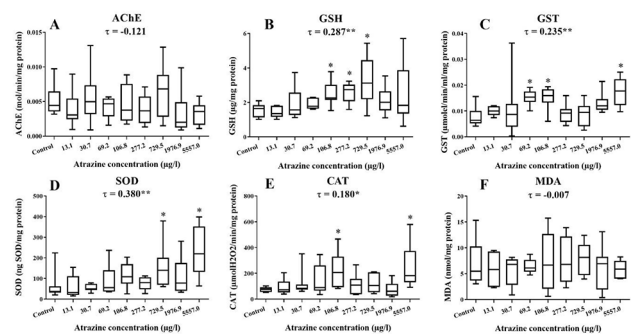
## Results and Discussion

Biomarker results from exposed *C. africana* are shown in Fig. 1 A–F, with quantified atrazine concentrations used as independent variables. The atrazine concentrations that were quantified were similar to those calculated as nominal concentrations. The corresponding Kendall Tau's correlation coefficients have been indicated, per biomarker. All significant differences in the box-and-whisker plots ( $p < 0.0063$ ) are related to the control group as differences between concentrations was not of primary interest. Acetylcholinesterase activity (Fig. 1 A) showed no pairwise significant differences ( $H(8) = 8.90$ ,  $p > 0.05$ ) or correlation between concentration and AChE activity,  $\tau = -0.121$ ,  $p > 0.05$ . The inhibition of AChE as a result of exposure to pesticides is well documented with the most significant inhibitors are known to be carbamate and organophosphate pesticide groups, as their mode of action is to target these enzymes (Chambers et al. 2002). No significant response in AChE activity after atrazine exposure were found in similar studies by Steele et al. (2018) at 160  $\mu\text{g/l}$  in freshwater crayfish and Anderson and Lydy (2002) in *Hyaella azteca* at 200  $\mu\text{g/l}$ . Moreover, Jin-Clark et al. (2002) found no significant changes in AChE activity in midge larvae at 1 000  $\mu\text{g/l}$ .

Reduced glutathione levels (Fig. 1B) in *C. africana* showed a significantly increasing trend,  $\tau = 0.287$ ,  $p < 0.01$ . The Kruskal Wallis test indicated a statistically significant effect of concentration on GSH levels in *C. africana*,  $H(8) = 24.07$ ,  $p < 0.05$ . The concentrations 106.8, 277.2 and 729.5  $\mu\text{g/l}$  were found to be significantly different to the control ( $p < 0.0063$ ). Silveyra et al. (2018) found no significant difference in GSH levels in freshwater crayfish exposed

to 1 000  $\mu\text{g/l}$  atrazine but found a 17-fold increase ( $p < 0.01$ ) in GSH levels after exposure to 5 000  $\mu\text{g/l}$ . It is possible that below the 1000  $\mu\text{g/l}$  exposure concentration there were increased levels in GSH that could be similar to the results obtained for *C. africana*. Stara et al. (2018) found that exposure of *Cherax destructor* to 6.86  $\mu\text{g/l}$  and 1 210  $\mu\text{g/l}$  of atrazine for 7 days resulted in decreased GSH levels. Along with GSH, GST (Fig. 1 C) is also considered a Phase II biotransformation enzyme that acts on electrophilic compounds such as reactive oxygen species (ROS) (Griboff et al. 2014). The responses showed two concentration regions that had higher peak distribution with statistically significant differences from control found in organisms exposed to 69.2, 106.8 and 5557  $\mu\text{g/l}$  ( $p < 0.0063$ ). Although there was a decrease in GST levels in organisms exposed to concentrations between 106.8 and 5557 there was a statistically significant correlation between exposure concentration and GST response,  $\tau = 0.235$ ,  $p < 0.01$ .

Antioxidant enzyme SOD (Fig. 1D) showed a statistically significant positive correlation between enzyme activity and atrazine concentration ( $\tau = 0.180$ ,  $p < 0.05$ ), reflected in the Kruskal Wallis test,  $H(8) = 24.68$ ,  $p < 0.05$ . Organisms exposed to 729.5 and 5557  $\mu\text{g/l}$  atrazine showed significantly higher levels of SOD than organisms in the control group ( $p < 0.0063$ ). The increasing trend indicates that there is a need for the body to upregulate its defences against possible superoxide anion radical formation. Atrazine has been found to increase SOD in freshwater shrimp (380  $\mu\text{g/l}$ ) and crayfish (1210  $\mu\text{g/l}$ ) after 14 and 21 days respectively (Griboff et al., 2014; Stara et al., 2018). The upregulation in the formation of SOD was attributed to the body's adaptive mechanisms in dealing with ROS. The superoxide anion radical is formed naturally in the body of most organisms



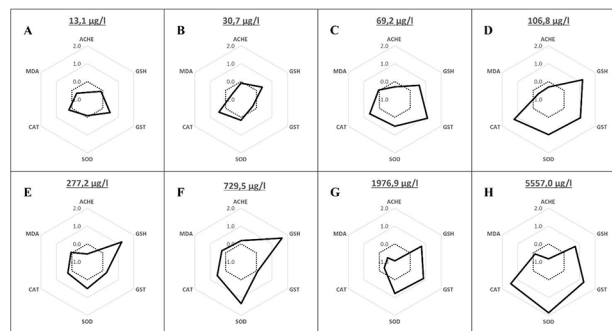
**Fig. 1** Box-and-whisker plots representing median, interquartile range and 5–95 percentile of individual biomarkers with the associated Kendall Tau's correlation coefficient (?) according to different exposure concentrations of atrazine. Statistically significant differences for box and whisker plots compared to control are indicated by either a single asterisk ( $p < 0.0063$ ) and significant correlation a single asterisk ( $p < 0.05$ ) or double asterisk ( $p < 0.01$ ). AChE – acetylcholinesterase; GSH – reduced glutathione; GST – glutathione-S-transferase; SOD – superoxide dismutase; CAT – catalase; MDA – malondialdehyde



and is reactive due to its unpaired electron (Fernández et al., 2009). Catalase activity (Fig. 1E) in the test organisms was influenced by the exposure concentration,  $H(8) = 20.37, p < 0.05$ . Groups that were significantly different from the control were exposed to 106.8 and 5557  $\mu\text{g/l}$  atrazine, ( $p < 0.0063$ ). Following the dismutation of the superoxide anion radical, hydrogen peroxide is formed, which can also cause oxidative damage. Catalase neutralises hydrogen peroxide by converting it to water and oxygen. Similar to SOD, a positive correlation between CAT activity and exposure concentrations was also noted,  $\tau = 0.180, p < 0.05$ . This is expected as there is usually a relationship in the SOD – CAT system (Hong et al., 2018). Malondialdehyde is formed when lipid structures undergo oxidation from ROS. If there is enough protection from the antioxidant system, this can protect the organisms macromolecular structures and prevent oxidative damage (Nikinmaa, 2014). Lipid peroxidation was measured in the test organisms as MDA (Fig. 1F). Kruskal Wallis, and Kendall Tau correlation results show no indication of an increasing or decreasing trend in MDA content,  $H(8) = 1.53, p > 0.05; \tau = -0.007, p > 0.05$ , possibly indicating that the oxidative stress enzymes were still coping to prevent oxidative damage.

The original IBR was updated as it could produce different results depending on how the biomarkers were ordered in the analysis (Beliaeff and Burgeot 2002). The IBRv2 gives an overall score based on the biomarker response’s deviation from a control group (Sanchez et al. 2013). Table 1 provides the reference deviation scores as well as the final IBRv2 score, with Fig. 2 graphically representing excitation and inhibition of the biomarkers based on the deviation from the reference (zero mark). The scores ranged from 1.73 in the second lowest concentration to 6.25 in the highest concentration.

The radar plots (Fig. 2 A-H) are most dissimilar from the control group at concentrations 106.8  $\mu\text{g/l}$  (Fig. 2 D) and 5557  $\mu\text{g/l}$  (Fig. 2 H), where the calculated IBRv2 scores were the highest. There are two peaks identified in the distribution of response scores with the first peak at 106.8  $\mu\text{g/l}$



**Fig. 2** Radar plots showing IBRv2 scores per exposure concentration. Area below zero indicates inhibition compared to the reference group and above zero indicates stimulation compared to the reference group. AChE – acetylcholinesterase; GSH – reduced glutathione; GST – glutathione-S-transferase; SOD – superoxide dismutase; CAT – catalase; MDA – malondialdehyde

and the second peak at 5557  $\mu\text{g/l}$ . The radar plots indicate that the most significant deviation at these concentrations was seen in the oxidative stress biomarkers GSH, GST, SOD and CAT. This is a reflection from the univariate analysis as the Kruskal Wallis test indicated that these two groups were the most frequently flagged as statistically significantly different from the control group.

This standardized IBRv2 approach has been used successfully in assessing the impacts of different stressors on a variety of organisms under both laboratory and field studies. Dahms-Verster et al. (2020) found increased IBRv2 scores in frogs (*Xenopus laevis*) exposed to higher concentrations of vanadium while Li et al. (2018) found increased IBRv2 values in field sampled snails from the Taihu Lake in China, aiding in the identification of sites where organisms were under greater stress.

### Conclusions

In this study, atrazine showed a significant effect on the biomarker responses in *C. africana*, particularly at 106.8

**Table 1** Reference deviation values (A) for each biomarker. Negative values for each biomarker indicate inhibition from the reference group, while positive values indicate stimulation compared to the reference group. Final IBRv2 scores for each concentration are provided with higher scores indicating a larger deviation from the reference group. AChE – acetylcholinesterase; GSH – reduced glutathione; GST – glutathione-S-transferase; SOD – superoxide dismutase; CAT – catalase; MDA – malondialdehyde

Biomarkers	Atrazine exposure concentrations								
	13.1 $\mu\text{g/l}$	30.7 $\mu\text{g/l}$	69.2 $\mu\text{g/l}$	106.8 $\mu\text{g/l}$	277.2 $\mu\text{g/l}$	729.5 $\mu\text{g/l}$	1976.9 $\mu\text{g/l}$	5557 $\mu\text{g/l}$	IBRv2
AChE	-0.60	-0.09	-0.30	-0.31	-0.56	0.18	-0.94	-0.83	
GSH	0.48	-0.24	1.11	1.06	0.23	0.07	0.86	1.27	
GST	0.19	0.43	0.62	1.23	0.26	0.54	-0.32	1.45	
SOD	-0.09	0.17	0.50	0.97	0.49	1.34	0.77	1.85	
CAT	-0.11	0.37	0.58	1.19	1.22	1.65	0.73	0.72	
MDA	-0.31	-0.42	0.04	-0.36	0.05	0.24	-0.54	-0.12	
<b>IBRv2</b>	<b>1.78</b>	<b>1.73</b>	<b>3.15</b>	<b>5.13</b>	<b>2.82</b>	<b>4.02</b>	<b>4.17</b>	<b>6.25</b>	

and 5557 µg/l exposure concentrations. Short term (acute) testing is valuable to see immediate stress that organisms are under. The results from this study show that there is a clear oxidative stress response from the test organisms. The use of the IBRv2 indicates graphically how these organisms' responses are deviating from their normal level. The integration of all biochemical responses into a standardised index allows for easy interpretation and should be included in multi-biomarker studies. This study reveals that although ATZ is said to have little effect on aquatic organisms (e.g. USEPA limit for acute protection of aquatic life is 350 µg/l), there are nuanced effects that may result in longer term impacts. Future investigation into the gene expression that leads to the up regulation of certain oxidative stress responses would give further insight into how these responses are formed. This in turn would allow for the determination of adverse outcomes pathways (AOPs).

**Acknowledgements** We would like to the National Research Foundation of South Africa for the financial support granted to GR Jansen van Rensburg (NRF scarce skills scholarship, Grant 101,325). We would also like to thank the University of Johannesburg and the Water Research Group (WRG) at the North-West University for their support during this study.

## References

- Anderson TD, Lydy MJ (2002) Increased toxicity to invertebrates associated with a mixture of atrazine and organophosphate insecticides. *Environ Toxicol Chem* 21:1507–1514. <https://doi.org/10.1002/etc.5620210724>
- Ansara-Ross T, Wepener V, van den Brink P, Ross M (2012) Pesticides in South African fresh waters. *Afr J Aquat Sci* 37. <https://doi.org/10.2989/16085914.2012.666336>
- Atwood D, Paisley-Jones C (2017) Pesticides industry sales and usage: 2008–2012 market estimates. United States Environmental Protection Agency (USEPA), Washington, D.C.
- Beliaeff B, Burgeot T (2002) Integrated biomarker response: A useful tool for ecological risk assessment. *Environ Toxicol Chem* 21:1316–1322. <https://doi.org/10.1002/etc.5620210629>
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254. [https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3)
- Canada Health (1993) Guidelines for Canadian drinking water quality, guideline technical document, atrazine. Health Canada, Ottawa, Canada
- Chambers JE, Boone JS, Carr RL, Chambers HW, Straus DL (2002) Biomarkers as Predictors in Health and Ecological Risk Assessment. *Hum Ecol Risk Assess An Int J* 8:165–176. <https://doi.org/10.1080/20028091056809>
- Cohen G, Dembiec D, Marcus J (1970) Measurement of catalase activity in tissue extracts. *Anal Biochem* 34:30–38. [https://doi.org/10.1016/0003-2697\(70\)90083-7](https://doi.org/10.1016/0003-2697(70)90083-7)
- Cohn VH, Lyle J (1966) A fluorometric assay for glutathione. *Anal Biochem* 14:434–440. [https://doi.org/10.1016/0003-2697\(66\)90286-7](https://doi.org/10.1016/0003-2697(66)90286-7)
- Dabrowski JM (2015) Development of pesticide use maps for South Africa. *S Afr J Sci* 111:1–7. <https://doi.org/10.17159/sajs.2015/20140091>
- Dahms-Verster S, Nel A, van Vuren JHJ, Greenfield R (2020) Biochemical responses revealed in an amphibian species after exposure to a forgotten contaminant: An integrated biomarker assessment. *Environ Toxicol Pharmacol* 73. <https://doi.org/10.1016/j.etap.2019.103272>
- Davies P, Cook L, Barton J (1994) Triazine herbicide contamination of Tasmanian streams: sources, concentrations and effects on biota. *Mar Freshw Res* 45:209. <https://doi.org/10.1071/MF9940209>
- De A, Bose R, Kumar A, Mozumdar S (2014) Worldwide pesticide use. Targeted Delivery of Pesticides Using Biodegradable Polymeric Nanoparticles. Springer, New Delhi, India, pp 5–6. [https://doi.org/10.1007/978-81-322-1689-6\\_2](https://doi.org/10.1007/978-81-322-1689-6_2)
- De Grave S, Smith KG, Adeler NA, Allen DJ, Alvarez F, Anker A, Cai Y, Carrizo SF, Klotz W, Mantelatto FL, Page TJ, Shy J-Y, Villalobos JL, Wowor D (2015) Dead shrimp blues: a global assessment of extinction risk in freshwater shrimps (Crustacea: Decapoda: Caridea). *PLoS ONE* 10:e0120198. <https://doi.org/10.1371/journal.pone.0120198>
- de Mazancourt V, Klotz W, Marquet G, Mos B, Rogers DC, Keith P (2019) The complex study of complexes: The first well-supported phylogeny of two species complexes within genus *Caridina* (Decapoda: Caridea: Atyidae) sheds light on evolution, biogeography, and habitat. *Mol Phylogenet Evol* 131:164–180. <https://doi.org/10.1016/j.ympev.2018.11.002>
- Del Maestro RF, McDonald W (1985) Oxidative enzymes in tissue homogenates. In: Greenwald RA (ed) *Handbook of Methods for Oxygen Radical Research*. CRC Press, Boca Raton, pp 291–296
- Della-Flora A, Becker RW, Ferrão MF, Toci AT, Cordeiro GA, Boroski M, Sirtori C (2018) Fast, cheap and easy routine quantification method for atrazine and its transformation products in water matrixes using a DLLME-GC/MS method. *Anal Methods* 10:5447–5452. <https://doi.org/10.1039/c8ay02227e>
- Du Preez LH, van Jansen PJ, Jooste AM, Carr JA, Giesy JP, Gross TS, Kendall RJ, Smith EE, Van Der Kraak G, Solomon KR (2005) Seasonal exposures to triazine and other pesticides in surface waters in the western Highveld corn-production region in South Africa. *Environ Pollut* 135:131–141. <https://doi.org/10.1016/j.envpol.2004.09.019>
- DWAF (1996) South African water quality guidelines: Volume 1 domestic use, Department of Water Affairs and Forestry. Department of Water Affairs and Forestry, Pretoria, South Africa
- Ellman GL, Courtney KD, Andres V, Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 7:88–95. [https://doi.org/10.1016/0006-2952\(61\)90145-9](https://doi.org/10.1016/0006-2952(61)90145-9)
- Fernández C, Ferreira E, San Miguel E, Fernández-Briera A (2009) Superoxide dismutase and catalase: tissue activities and relation with age in the long-lived species *Margaritifera margaritifera*. *Biol. Res.* 42, 57–68. <https://doi.org/S0716-97602009000100006>
- Graymore M, Stagnitti F, Allinson G (2001) Impacts of atrazine in aquatic ecosystems. *Environ Int* 26:483–495. [https://doi.org/10.1016/S0160-4120\(01\)00031-9](https://doi.org/10.1016/S0160-4120(01)00031-9)
- Griboff J, Morales D, Bertrand L, Bonansea RI, Monferrán MV, Asis R, Wunderlin DA, Amé MV (2014) Oxidative stress response induced by atrazine in *Palaemonetes argentinus*: The protective effect of vitamin E. *Ecotoxicol Environ Saf* 108:1–8. <https://doi.org/10.1016/j.ecoenv.2014.06.025>
- Habig W, Pabst M, Jakoby W (1974) Glutathione S transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* 249:7130–7139
- Hong Y, Yang X, Huang Y, Yan G, Cheng Y (2018) Assessment of the oxidative and genotoxic effects of the glyphosate-based herbicide roundup on the freshwater shrimp,

- Macrobrachium nipponensis*. Chemosphere 210:896–906. <https://doi.org/10.1016/j.chemosphere.2018.07.069>
- Jablonowski ND, Schäffer A, Burauel P (2011) Still present after all these years: persistence plus potential toxicity raise questions about the use of atrazine. Environ Sci Pollut Res 18:328–331. <https://doi.org/10.1007/s11356-010-0431-y>
- van Jansen G, Bervoets L, Smit NJ, Wepener V, van Vuren J (2020) Biomarker Responses in the Freshwater Shrimp *Caridina nilotica* as Indicators of Persistent Pollutant Exposure. Bull Environ Contam Toxicol 104:193–199. <https://doi.org/10.1007/s00128-019-02773-0>
- Jin-Clark Y, Zhu K, Lydy M (2002) Effects of atrazine and cyanazine on chlorpyrifos toxicity in Chironomus tentans (diptera: Chironomidae). Environ Toxicol Chem 21:598–603
- Li Q, Wang M, Duan L, Qiu Y, Ma T, Chen L, Breitholtz M, Bergman A, Zhao J, Hecker M, Wu L (2018) Multiple biomarker responses in caged benthic gastropods *Bellamyia aeruginosa* after in situ exposure to Taihu Lake in China. Environ Sci Eur 30:34. <https://doi.org/10.1186/s12302-018-0164-y>
- Miller JN, Miller JC (2010) Statistics and chemometrics for analytical chemistry, 6th edn. Pearson Education, Harlow
- Muller WJ, Slaughter AR, Ketse N, Davies-coleman HD, Kock EDE, Palmer CG (2011) Development of chronic toxicity test methods for selected indigenous riverine macroinvertebrates (No. 1313/3/11). Water Research Commission. <https://doi.org/978-1-4312-0167-9>
- Munch J (1995) Determination of chlorinated pesticides, herbicides, and organohalides by liquid-solid extraction and electron capture gas chromatography. U. S. Environmental Protection Agency. Cincinnati
- Nikinmaa M (2014) An introduction to aquatic toxicology. Academic Press, Elsevier, Waltham, Massachusetts, pp 221–232
- OECD (2019) OECD guideline for the testing of chemicals. Test guideline 203. Fish, acute toxicity test. Organisation for Economic Co-operation and Development. <https://doi.org/10.1787/9789264070684-en>
- Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351–358. [https://doi.org/10.1016/0003-2697\(79\)90738-3](https://doi.org/10.1016/0003-2697(79)90738-3)
- Richard J, Clark PF (1995) African *Caridina* (Crustacea: Decapoda: Caridea: Atyidae): redescrptions of *C. africana* Kingsley, 1882, *C. togoensis* Hilgendorf, 1893, *C. natalensis* Bouvier, 1925 and *C. roubaudi* Bouvier, 1925 with descriptions of 14 new. Zootaxa. Magnolia Press, Auckland, New Zealand. <https://doi.org/10.11646/zootaxa.1995.1.1>
- Rimayi C, Odusanya D, Weiss JM, de Boer J, Chimuka L (2018) Seasonal variation of chloro-s-triazines in the Hartbeespoort Dam catchment, South Africa. Sci Total Environ 613–614:472–482. <https://doi.org/10.1016/j.scitotenv.2017.09.119>
- Sanchez W, Burgeot T, Porcher J-M (2013) A novel “Integrated Biomarker Response” calculation based on reference deviation concept. Environ Sci Pollut Res 20:2721–2725. <https://doi.org/10.1007/s11356-012-1359-1>
- Sass JB, Colangelo A (2006) European Union bans atrazine, while the United States negotiates continued use. Int J Occup Environ Health 12:260–267. <https://doi.org/10.1179/oeh.2006.12.3.260>
- Schank JC, Koehnle TJ (2009) Pseudoreplication is a pseudoproblem. J Comp Psychol 123:421–433. <https://doi.org/10.1037/a0013579>
- Silveyra GR, Silveyra P, Vatnick I, Medesani DA, Rodríguez EM (2018) Effects of atrazine on vitellogenesis, steroid levels and lipid peroxidation, in female red swamp crayfish *Procambarus clarkii*. Aquat Toxicol 197:136–142. <https://doi.org/10.1016/j.aquatox.2018.02.017>
- Stara A, Kouba A, Velisek J (2018) Biochemical and histological effects of sub-chronic exposure to atrazine in crayfish *Cherax destructor*. Chem Biol Interact 291:95–102. <https://doi.org/10.1016/j.cbi.2018.06.012>
- Steele AN, Belanger RM, Moore PA (2018) Exposure through runoff and ground water contamination differentially impact behavior and physiology of crustaceans in fluvial systems. Arch Environ Contam Toxicol 75:436–448. <https://doi.org/10.1007/s00244-018-0542-x>
- Strahan RF (1982) Assessing Magnitude of Effect from Rank-Order Correlation Coefficients. Educ Psychol Meas 42:763–765. <https://doi.org/10.1177/001316448204200306>
- Tincani FH, Galvan GL, Marques AEM, Santos GS, Pereira LS, da Silva TA, Silva de Assis HC, Barbosa RV, Cestari MM (2017) Pseudoreplication and the usage of biomarkers in ecotoxicological bioassays. Environ Toxicol Chem 36:2868–2874. <https://doi.org/10.1002/etc.3823>
- USEPA (2007) Atrazine Chemical Summary. United States Environmental Protection Agency, Washington, D.C., United States of America
- USEPA (2002) Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms (No. 821-R-02-012). United States Environmental Protection Agency, Washington D.C., United States of America
- Zhang W (2018) Global pesticide use: Profile, trend, cost / benefit and more. Proc. Int. Acad. Ecol. Environ. Sci. 8, 1–27

**Publisher's Note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.