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Oxidative Stress Responses and Antioxidant Gene Regulation in *Daphnia pulex* Following Chronic Benzyl Alcohol Exposure

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ABSTRACT

Benzyl alcohol was evaluated for its short-term and long-term toxicity in Daphnia pulex, with an emphasis on oxidative stress biomarkers and antioxidant-related gene expression. Acute toxicity was evaluated over 48 h to estimate the LC50, and two sublethal concentrations (11.5 and 23 mg/L) were subsequently selected for a 21-day exposure. Activities of catalase (CAT), superoxide dismutase (SOD), and glutathione S-transferase (GST), and the expression profiles of cat and sod were measured, and associations between biochemical and molecular responses were examined. Mortality increased with concentration, yielding a 48 h LC_{50} of 229.68 mg/L. Prolonged exposure produced contrasting responses moderate stress stimulated antioxidant enzymes and gene expression, whereas higher exposure suppressed enzymatic activity, depleted glutathione reserves, and reduced transcriptional responses. Enzyme activities were positively correlated with gene expression, indicating coordinated regulation of the antioxidant defense system. These patterns indicate that oxidative imbalance develops at concentrations well below lethal thresholds and precedes overt mortality, supporting the use of biochemical and molecular biomarkers as sensitive indicators of chronic toxic stress in freshwater invertebrates.

HIGHLIGHTS

- Benzyl alcohol caused moderate acute toxicity in *Daphnia pulex*.
- Chronic exposure triggered biphasic antioxidant enzyme response.
- Glutathione depletion indicated a progressive oxidative imbalance.
- Cat and sod genes showed dose-dependent transcriptional regulation.
- Biomarkers were more sensitive indicators than mortality endpoints.

1. INTRODUCTION

Organic contaminants and other environmental stressors have become increasingly recognised for their potential to influence ecologically significant changes in aquatic ecosystems, where aquatic life is always under a combination of natural and anthropogenic pressures (Al-Jaff, 2021). For instance, research conducted on water fleas, *Daphnia pulex* demonstrated their biological responses to a combination of environmental stressors, including temperature, salinity, and nutrient levels, and cues for waterborne contaminants, even when there was a lack of acutely toxic substances. The pervasiveness and ecological relevance of emerging contaminants and other environmental stressors in aquatic ecosystems have also been emphasized by the fact that aquatic life is more likely to respond to long-term, low-level exposures rather than to acutely toxic substances. Aquatic life is likely to respond with physiological, morphological, and life-history alterations through long-term, low-level exposure to environmental stressors. As a result, the response to sublethal toxicity is considered to have ecological relevance for aquatic life (McKnight et al., 2023).

Benzyl alcohol is an aromatic alcohol widely used as a solvent and preservative in pharmaceutical, cosmetic, household, and sanitizer-related formulations. Owing to these widespread applications, its release into wastewater streams and subsequent entry into receiving freshwater systems are environmentally plausible (Manjunatha et al., 2021; Musee et al., 2023; Api et al., 2025). Environmental

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monitoring studies have reported the presence of benzyl alcohol in wastewater treatment plants at concentrations of 500-11,000 ng/L in influents and 150-400 ng/L in effluents, and it has also been detected in groundwater with a median concentration of 582 ng/L (Kong et al., 2016; Musee et al., 2023). Aquatic toxicity data further support its ecological relevance, with reported 24 h and 48 h EC50 values of 55 mg/L and 230 mg/L, respectively, for *Daphnia magna* (Musee et al., 2023). Previous studies in aquatic models, including zebrafish embryos, have also shown that benzyl alcohol can induce mortality, developmental abnormalities, and organ-level toxicity (Manjunatha et al., 2021). However, information remains limited regarding its long-term sublethal effects on oxidative stress biomarkers and antioxidant-related gene expression in freshwater invertebrates such as *Daphnia pulex*.

Daphnia species are widely recognized as important model organisms in ecology and freshwater ecotoxicology because of their ecological role in pelagic food webs, sensitivity to dissolved contaminants, short generation time, and suitability for mechanistic toxicity studies. In addition, their usefulness in linking molecular and biochemical responses to organism- and population-level effects has made them valuable models for evaluating contaminant-induced stress in aquatic systems (Ebert, 2022; Reilly et al., 2023). Therefore, *Daphnia pulex* was selected in the present study as a suitable freshwater model for assessing chronic sublethal oxidative stress responses to benzyl alcohol exposure.

Oxidative stress is commonly defined as an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense system's capacity to detoxify them, leading to cellular oxidative damage. In aquatic invertebrates, environmental stressors have been shown to disrupt antioxidant defenses through altered ROS production and impaired redox homeostasis, thereby supporting oxidative stress as a central mechanism of toxicity (Atli and Sevgiler, 2024; Esterhuizen et al., 2024).The antioxidant defense system also includes antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione-S-transferase (GST) which have also been reported to be highly sensitive to changes due to the presence of toxicants as well as changes in the homeostasis of the cells. Other non-enzymatic antioxidants, such as reduced glutathione have also been widely measured in combination with antioxidant enzymes to evaluate antioxidant status. The difference in antioxidant defense systems has also been widely used to measure the early changes of stress in the field of ecotoxicology, where changes due to the presence of toxicants can be clearly detected before any toxicity and/or cell death are evident (Atli and Sevgiler, 2024; Mahaye and Musee, 2022). Although antioxidant enzymes such as CAT and SOD are widely recognized as sensitive biomarkers of chemical stress in Daphnia, studies specifically addressing long-term benzyl alcohol-induced enzymatic responses in freshwater cladocerans remain very limited. Recent experimental studies on *Daphnia pulex* provide quantitative evidence of the sensitivity of CAT and SOD to chemical stressors. Under chronic nanoplastic exposure (0.1-1.0 mg/L for 21 days) SOD expression was significantly upregulated at 0.5 and 1.0 mg/L whereas CAT expression was significantly downregulated at 0.5-2.0 mg/L compared to controls ($p<0.05$) (Liu et al., 2018). Field investigations in cyanobacteria-impacted freshwater systems reported catalase activities of 25.21 U/mg protein in contaminated reservoirs compared to 14.13 U/mg protein in reference lakes, with seasonal maxima reaching 40.94 U/mg protein (Wojtal-Frankiewicz et al., 2013). Acute exposure to cyanotoxin mixtures resulted in an approximately 28% reduction in CAT activity within 24 h at 100 µg/L (Esterhuizen-Londt et al., 2016). Similarly, combined copper (40 µg/L) and cadmium (20 µg/L) exposure reduced SOD activity from approximately 6 U/min/mg protein in controls to about 2 U/min/mg protein after 24 h and to 2.5-3.0 U/min/mg protein after 48 h (Sadeq, 2018).

The gene expression response was found to be an essential mechanism in the adaptation of *Daphnia* spp. to oxidative stress, where oxidative stress is often the result of environmental contaminants, Antioxidant-related gene categories in *Daphnia* spp., including the gene categories of cat and sod, were found to be responsive to oxidative stress, as these gene categories play an essential role in the regulation of the antioxidant defense system in aquatic animals (Kim et al., 2011; Wang et al., 2019). Such changes in the expression levels are often accompanied by similar changes in the corresponding enzymatic activities, underscoring the significance of the transcriptional control process (Sonmez et al., 2025). Combining molecular biomarkers, including gene expression profiles, with the measurement of the parameters of the antioxidant defense system would enable a wider approach to the assessment of oxidative stress in *Daphnia* spp., as gene expression changes can occur

either prior to or in conjunction with enzymatic changes, which would greatly strengthen the ecotoxicological assessment results (Wang et al., 2019; Guevara et al., 2024). Nevertheless, comparable long-term transcriptional evidence for benzyl alcohol exposure in *Daphnia pulex* remains scarce, which further supports the need for the present investigation. The above trends have been observed in Iraqi freshwater species exposed to organic/petroleum pollutants. According to the literature, the activity of antioxidant enzymes in invertebrates exposed to organophosphate pollutants is considerably affected by the rise in oxidative stress levels, which negatively influences the activity of the aforementioned enzymes (Al-Fanharawi et al., 2019). Fish species in polluted aquatic cultures experience remarkable changes in the activity of antioxidant enzymes due to long-term stress caused by pollutants (Alkatrani et al., 2024). Oxidative stress biomarkers respond intensively to hydrocarbon pollutants, which makes them applicable for the early detection of oil pollution using the sublethal method (Abdulameer and Hussein, 2023). Chronic pollutant exposure may alter antioxidant enzyme activity in a concentration and duration-dependent manner. Antioxidant enzymes may initially increase as an adaptive response to elevated ROS production, whereas prolonged or stronger stress can reduce enzyme activity as a result of impaired antioxidant regulation, reduced protective capacity, or partial homeostatic adjustment (Mustafa et al., 2024).

Although benzyl alcohol has been investigated in aquatic models and acute toxicity data are available for *Daphnia magna* (Manjunatha et al., 2021; Musee et al., 2023), evidence specifically addressing its long-term sublethal effects on oxidative stress biomarkers and antioxidant-related gene expression in *Daphnia pulex* is still scarce. Therefore, the present study aimed to evaluate the acute toxicity of benzyl alcohol and to investigate its long-term sublethal effects on oxidative stress biomarkers and antioxidant-related gene expression in *Daphnia pulex*.

2. METHODOLOGY

2.1 Chemical test

Benzyl alcohol (>99% purity; C_7H_8O; molecular weight: 108.14 g/mol; CAS No. 100-51-6) was obtained from Loba Chemie, India, and used without further purification. A stock solution was freshly prepared in distilled water and stored at 4°C until use. Working exposure solutions were prepared by diluting the stock solution with the exposure medium to the required concentrations. Fresh exposure media were renewed every 48 h during the chronic exposure period. Concentrations are expressed as mg/L. The exposure concentrations of benzyl alcohol were derived from the 48 h LC_{50} value obtained in the present acute toxicity test using *Daphnia pulex*. For the 21-day chronic experiment, 5% and 10% of the LC_{50} were selected as sublethal exposure levels, corresponding to 11.5 and 23 mg/L, respectively. These LC_{50}-derived fractions were selected to represent low and higher sublethal exposure levels for evaluating biochemical and molecular biomarker responses, consistent with previous *Daphnia* studies using LC_{50} based fractions in chronic and molecular toxicity assessments (Bang et al., 2015; Poynton et al., 2011).

2.2 Test organism and culture conditions

The test organism used was *D. pulex*, a freshwater cladoceran. The test cultures were maintained under laboratory-controlled conditions and were exposed to aerated water prepared as reconstituted ASTM hard water (ASTM International, 2014) at a constant temperature of 20±1°C, a lighting period of 16 hours and a dark period of 8 hours, and with an aqueous solution of the green algae *Scenedesmus* sp. at approximately 1×10^5 cells/mL to ensure that the test animals were never food limited. The exposure medium was renewed every 48 h to maintain water quality throughout the experiment. All acute and chronic exposure experiments were conducted using *Daphnia pulex* neonates less than 24 h old. Before starting the test, the test organisms were subjected to acclimatization under laboratory conditions.

2.3 Acute toxicity and selection of sublethal concentrations

A 48 h acute toxicity test was conducted following OECD Test Guideline 202 (OECD, 2004) to determine the LC_{50} of benzyl alcohol and to select sublethal concentrations for the chronic exposure experiment. The assay was performed using *Daphnia pulex* neonates less than 24 h old. Benzyl alcohol was tested at concentrations of 150, 180, 210, 240, and 270 mg/L, in addition to a control group without benzyl alcohol. Each concentration consisted of three replicates with 10 individuals per replicate (n=30). In the acute test carried out to determine the LC_{50} values of benzyl alcohol, individuals showing no movement after gentle agitation were considered dead. LC_{50}

values were estimated by probit analysis using log-transformed concentrations, following the statistical method described by Finney (1971). The calculated 48 h LC_{50} was 230 mg/L. Two sublethal concentrations were then selected for the chronic test at 5% and 10% of the calculated LC_{50}, corresponding to 11.5 and 23 mg/L, respectively.

2.4 Chronic exposure setup and sampling strategy

Replicate beakers were prepared to correspond to each treatment group: control (0 mg/L), treatment group of 11.5 mg/L, and treatment group of 23 mg/L. Each replicate initially contained 20 neonates (<24 h old), which were maintained for 21 days under chronic exposure conditions, with three independent biological replicates per treatment (n=60 per group). Organism density did not exceed one individual per 10 mL. The exposure medium was renewed every 48 h under semi-static conditions using freshly prepared nominal concentrations to help maintain benzyl alcohol exposure levels and water quality throughout the chronic exposure period. Exposure vessels were kept partially covered to reduce possible evaporative loss and concentration fluctuation. At the end of the exposure period, *D. pulex* individuals were collected separately for biochemical and gene expression analyses (RT-qPCR).

2.5 Antioxidant enzyme and oxidative stress biomarkers

After 21 days of exposure to benzyl alcohol, *Daphnia pulex* samples were collected and homogenized in ice-cold phosphate buffer (100 mM, pH 7.4) at a ratio of 1:4 (w/v). The homogenates were centrifuged at 10,000 × g for 10 min at 4°C, and the resulting supernatants were used for subsequent biochemical analyses (Peppos et al., 2011). To evaluate oxidative stress responses in the exposed *Daphnia* populations, CAT, SOD, and GST activities were determined spectrophotometrically using the methods of Aebi (1984), Beauchamp and Fridovich (1971), and Habig et al. (1974), respectively. Reduced glutathione (GSH) content was measured colorimetrically according to Ellman (1959). Total protein concentration was determined using the Bradford method (Bradford, 1976), with bovine serum albumin as the standard. Enzyme activities and GSH content were normalized to total protein content and expressed per mg protein.

2.6 Gene expression analysis by RT-qPCR

For the purpose of gene expression analysis, approximately 10 adult *D. pulex* individuals were pooled per biological replicate, with three independent replicates (n=3) to prepare the samples. These were snap-frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted from the samples using the TRIzol reagent according to the manufacturer's protocol. RNA integrity and quality were verified prior to cDNA synthesis. Reverse transcription was conducted using commercial reverse transcription kits according to the manufacturer's protocol, using a reaction volume of 20 µL. RT-qPCR was conducted on the extracted cDNA samples using the CFX96 Real-Time PCR System (Bio-Rad, USA) to quantify the expression of the cat and sod genes in the samples. The primers were specific to the cat and sod genes of *D. pulex* samples, and the primer sequences and annealing temperatures are presented in Table 1.

Table 1. Primer sequences and annealing temperatures used for RT-qPCR analysis in *D. pulex*

Gene	Primer	Sequence (5′-3′)	Annealing temperature (°C)
cat	Forward	TCCATTTGAACTTTTCTGCTTC	57.6
	Reverse	CCGACCAAGGGATTCGTAAT	57.6
sod	Forward	ATTGCTTTGTTGGTCTTGATGG	58.0
	Reverse	CCTTGCGGGGGTTGTAGTG	58.0
tbp	Forward	GCTGGCCATAGTGATCTTGG	59.0
	Reverse	CAGGAGTGATGCTGCTGTTG	59.0

The reference gene tbp was selected a priori for RT-qPCR normalization based on previous reference-gene evaluation studies in *Daphnia* species (Heckmann et al., 2006; Schwarzenberger et al., 2009; Spanier et al., 2010). This selection was further supported by the relatively consistent Cq values observed across control and treated samples under the present experimental conditions. Primer specificity was assessed using melt-curve analysis, and relative

expression levels of cat and sod were calculated using the $2^{-}\Delta\Delta Ct$ method with tbp as the reference gene.

2.7 Statistical analysis

Data were presented as mean±standard deviation (SD) of three biological replicates (n=3). For RT-qPCR data, technical replicates were averaged within each biological replicate, and statistical analysis was performed using ΔCt values, while $2^{-}\Delta\Delta Ct$ fold-change values were used for graphical presentation. Normality and homogeneity of variances were assessed using the Shapiro-Wilk and Levene's tests, respectively. One-way analysis of variance (ANOVA) followed by Tukey's HSD post hoc test, was applied to compare differences among treatment groups. Different letters in the figures indicate statistically significant differences among treatments. All analyses were performed using IBM SPSS Statistics.

3. RESULTS

3.1 Acute toxicity (LC_{50})

The acute toxicity of benzyl alcohol was determined, and mortality of *D. pulex* increased with increasing exposure concentrations after 48 h. No mortality was observed in the control group maintained in ASTM-reconstituted water (0 mg/L). Exposure to 150 mg/L resulted in 8 deaths out of 30 individuals (26.7%). Mortality increased to 33.3% and 43.3% at 180 and 210 mg/L, respectively, and further increased to 50.0% and 63.3% at 240 and 270 mg/L. Probit analysis of the mortality data estimated a 48 h LC_{50} of 229.68 mg/L, which was used to select sublethal concentrations for the chronic exposure experiments, as summarized in Table 2.

The selected chronic concentrations (11.5 and 23 mg/L) correspond to approximately 5% and 10% of the calculated LC_{50}, respectively.

3.2 Antioxidant biomarkers

Chronic exposure of *D. pulex* to benzyl alcohol for 21 days resulted in significant concentration-related alterations in antioxidant and oxidative stress biomarkers, as shown in Figure 1.

Table 2. Acute toxicity of benzyl alcohol to *D. pulex* after 48 h of exposure, showing the number of exposed and dead organisms at each concentration and corresponding mortality percentages.

Concentration (mg/L)	Exposed (N)	Dead (48 h)	Mortality (%)
0 (Control)	30	0	0.0
150	30	8	26.7
180	30	10	33.3
210	30	13	43.3
240	30	15	50.0
270	30	19	63.3

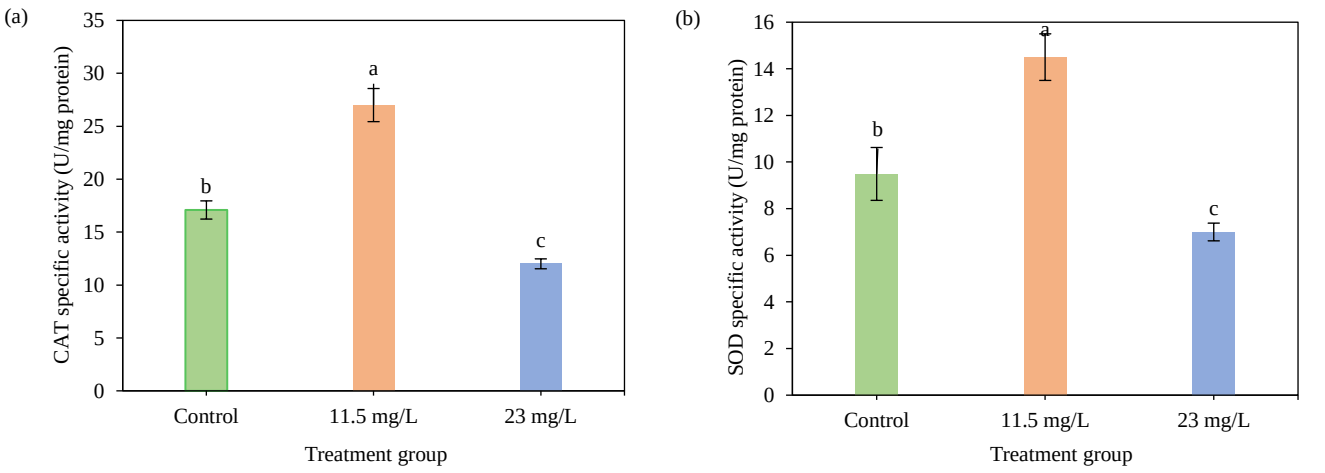


Figure 1. Effects of 21-day benzyl alcohol exposure on antioxidant biomarkers in *D. pulex*: (a) catalase (CAT), (b) superoxide dismutase (SOD), (c) glutathione S-transferase (GST), and (d) reduced glutathione (GSH). Data are presented as mean±SD (n=3). Different letters indicate significant differences among treatments (one-way ANOVA followed by Tukey's HSD test, $p<0.05$).

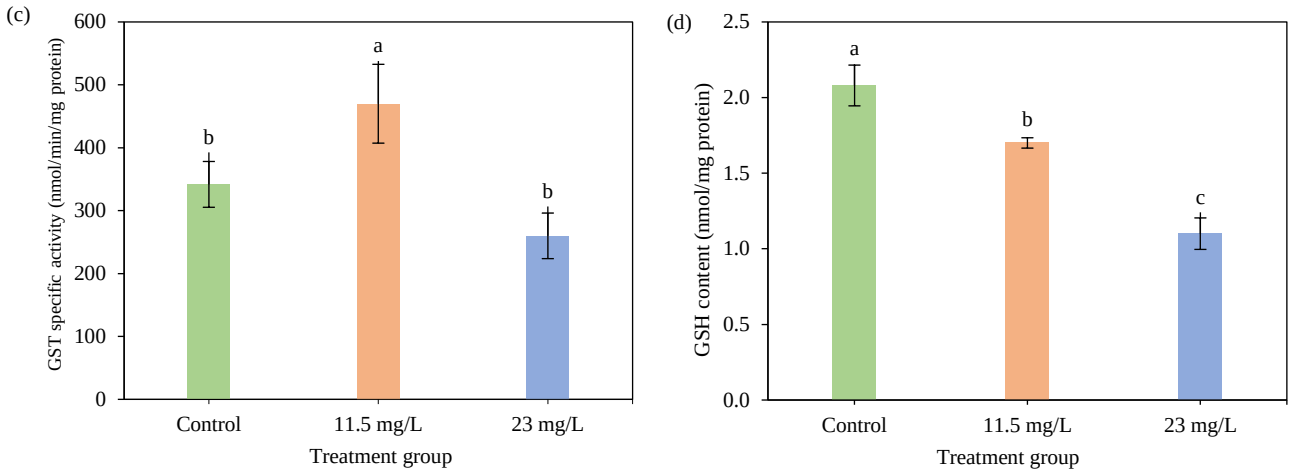


Figure 1. Effects of 21-day benzyl alcohol exposure on antioxidant biomarkers in *D. pulex*: (a) catalase (CAT), (b) superoxide dismutase (SOD), (c) glutathione S-transferase (GST), and (d) reduced glutathione (GSH). Data are presented as mean±SD (n=3). Different letters indicate significant differences among treatments (one-way ANOVA followed by Tukey's HSD test, $p<0.05$) (cont.).

Catalase (CAT) activity increased significantly from 17.09±0.86 U/mg protein in the control group to 27.00±1.57 U/mg protein at 11.5 mg/L, followed by a marked decrease to 12.00±0.47 U/mg/protein at 23 mg/L. Reduced glutathione (GSH) content showed a progressive decline from 2.08±0.14 nmol/ mg protein in the control to 1.70±0.03 nmol/ mg protein at 11.5 mg/L and further to 1.10±0.10 nmol mg/ protein at 23 mg/L. Glutathione S-transferase (GST) activity increased significantly at the lower concentration (470.00±62.63 nmol/min/mg protein) compared with the control (341.77±36.40 nmol/min/mg protein), but declined at 23 mg/L to 260.00±36.27 nmol/min/mg protein. Similarly, superoxide dismutase (SOD) activity rose from 9.49±1.13 U/mg protein in the control to 14.50±1.00 U/mg protein at 11.5 mg/L, then decreased significantly to 7.00±0.38 U/mg protein at 23 mg/L. Different superscript letters indicate statistically significant differences among treatments ($p<0.05$), demonstrating a biphasic antioxidant response characterized by enzymatic activation at moderate exposure and antioxidant impairment at higher benzyl alcohol concentrations.

3.3. Transcriptional responses of cat and sod genes

Chronic exposure of *D. pulex* to benzyl alcohol for 21 days induced significant, concentration-dependent regulation of antioxidant gene expression, as shown in Figure 2.

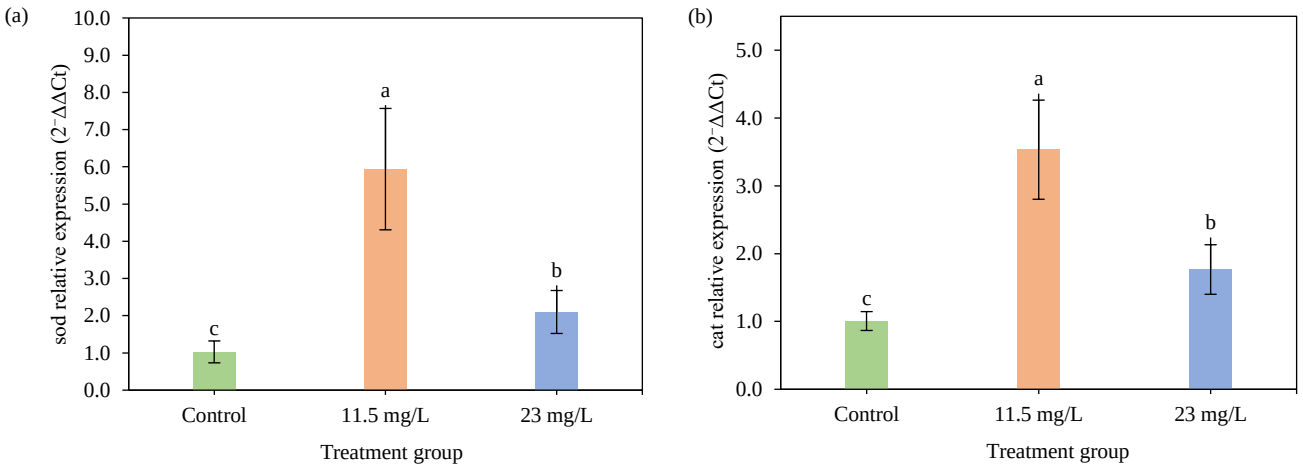


Figure 2. Relative expression of antioxidant-related genes: (a) sod and (b) cat in *D. pulex* after 21-day exposure to nominal benzyl alcohol concentrations.

Data are mean±SD (n=3 biological replicates). Different letters indicate significant differences among treatments (one-way ANOVA/Tukey's HSD, $p<0.05$); statistical analysis was performed using ΔCt values. Relative to the control group, cat expression increased significantly to 3.53±0.73-fold at 11.5 mg/L, followed by a decrease to 1.77±0.37-fold at 23 mg/L. However, cat expression at 23 mg/L remained significantly higher than the control and significantly lower than the 11.5 mg/L group. Similarly, sod expression increased significantly to 5.94±1.63-fold at 11.5 mg/L, then declined to 2.10±0.58-fold at 23 mg/L, remaining significantly above the control level but below the lower exposure group.

3.4 Correlation analysis

Results of Spearman correlation analysis showed that there were significant correlations between levels of antioxidant enzyme activity and expression response for antioxidant pathway genes in exposed populations of *D. pulex* in the benzyl alcohol exposure experiment (Figure 3).

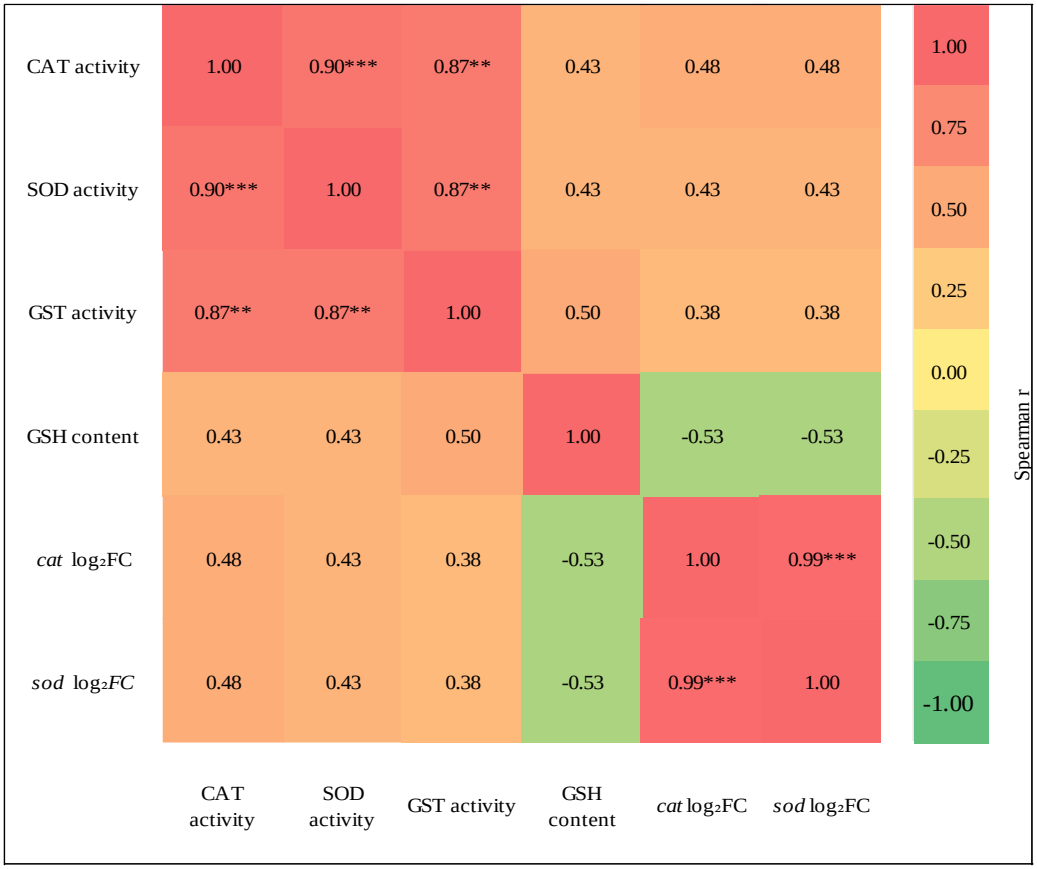


Figure 3. Spearman correlation heatmap showing the relationships between antioxidant enzyme activities (CAT, SOD, GST, and GSH) and the relative expression of cat and sod genes (log_2 fold change) in *D. pulex* after 21-day exposure to benzyl alcohol. Values represent Spearman correlation coefficients (r). Asterisks indicate statistically significant correlations (*p<0.05, **p<0.01, ***p<0.001).

Spearman correlation analysis showed strong positive relationships among antioxidant enzyme activities. CAT activity was strongly correlated with SOD activity ($r=0.90$, $p<0.001$), and both CAT and SOD activities were strongly correlated with GST activity ($r=0.87$, $p<0.01$). Regarding transcriptional responses, cat expression showed a near-perfect positive correlation with sod expression ($r=0.99$, $p<0.001$), suggesting coordinated regulation of antioxidant-related genes. Moderate positive associations were observed between antioxidant gene expression and enzyme activities, including associations between cat expression and CAT activity ($r=0.48$) and SOD activity ($r=0.43$). Similarly, sod expression showed moderate positive associations with CAT activity ($r=0.43$) and SOD activity ($r=0.48$). GSH content showed negative associations with cat and sod expression levels ($r=-0.53$), while its associations with CAT and SOD activities were weak.

4. DISCUSSION

4.1 Acute toxicity (LC_{50})

Although benzyl alcohol exhibited only moderate acute lethality (48 h LC_{50}=229.68 mg/L), chronic exposure produced pronounced enzymatic and transcriptional disturbances at concentrations nearly an order of magnitude lower than the lethal threshold. This discrepancy demonstrates that survival and mortality endpoints alone underestimate toxicological risk, as sublethal biochemical and physiological biomarkers in *Daphnia* frequently respond at concentrations far below those causing acute mortality (Mahaye and Musee, 2022). The acute mortality pattern was therefore less sensitive than chronic biomarker responses, indicating that oxidative stress-mediated physiological dysfunction precedes death and that biochemical endpoints provide earlier and more sensitive indicators of toxicity than lethality alone (Mahaye and Musee, 2022).Collectively, these findings highlight oxidative stress as the primary mechanistic pathway underlying both acute and chronic toxicity in *D. pulex*, consistent with broader evidence that chronic and multigenerational stress responses provide a more realistic estimate of ecological hazard (Livingstone, 2001; Regoli and Giuliani, 2014).

4.2 Antioxidant enzyme responses

After 21 days of exposure, clear changes were observed in the antioxidant defense system of *D. pulex*, and these changes depended strongly on benzyl alcohol concentration. At the intermediate level (11.5 mg/L), CAT, SOD, and GST activities increased compared with the control, suggesting that the animals activated their antioxidant defenses to cope with increased reactive oxygen species. Similar increases in enzyme activity have been reported in *Daphnia* exposed to sublethal contaminants (Esterhuizen et al., 2024; Queiroz and Torresi, 2025). This reaction may be linked to mitochondrial disruption and membrane damage, which can increase electron leakage and ROS production, thereby activating transcriptional and enzymatic defense mechanisms (Liu et al., 2021; Liu et al., 2022). At moderate concentrations, this pattern most likely reflects an early compensatory antioxidant response in which the defense system remained sufficiently functional to limit ROS-mediated damage and maintain redox balance. The situation changed, though, at the higher concentration (23 mg/L). Activities of CAT, SOD, and GST significantly decreased, and GSH levels steadily dropped. GSH depletion suggests that antioxidant reserves were being used up more quickly than they could be replenished because it is a significant non-enzymatic antioxidant and a necessary substrate for GST. Rather than indicating a simple loss of defense, this shift suggests that antioxidant regulation was altered under prolonged or stronger stress, with evidence of reduced protective capacity at the higher exposure level (De Felice et al., 2022; Mahaye and Musee, 2022). When combined, these findings reveal a common biphasic pattern, characterized by enzyme stimulation at the lower concentration and inhibition at the higher concentration, indicating a shift from an adaptive response to physiological impairment under long-term chemical stress. Antioxidant enzymes in *Daphnia* respond dynamically to oxidative stress. Ukhueduan et al. (2022) reported that higher SOD and CAT activities in *D. pulex* were associated with increased oxidative pressure, suggesting that enzyme elevation reflects a compensatory response to rising ROS levels. A similar trend was also described by Shen et al. (2019), where antioxidant enzymes were initially stimulated and later suppressed as contaminant stress intensified. However, unlike De Felice et al. (2022), who observed minimal changes in oxidative biomarkers under chronic nanoplastic exposure, the present findings indicate a clear redox disturbance induced by benzyl alcohol. Direct evidence on benzyl alcohol-induced antioxidant responses in aquatic organisms is still limited. However, its release through household and commercial waste streams, together with its detection in groundwater, supports its environmental relevance (Corcoran and Ray, 2014; Kong et al., 2016). In zebrafish embryos, benzyl alcohol has been reported to cause mortality, delayed hatching, cardiac defects, apoptosis, liver inhibition, and neuronal disruption, indicating potential toxicity to aquatic organisms (Manjunatha et al., 2021). Therefore, the antioxidant changes observed in the present study may represent early sublethal responses that could affect the physiological condition of freshwater organisms before mortality occurs (Musee et al., 2023).

4.3 Gene expression responses

Our understanding of the biochemical alterations caused by long-term exposure to benzyl alcohol has improved thanks to gene expression analysis. In general, the expression patterns of sod and cat mirrored the trend seen in enzyme activity. In comparison to the control group, sod expression rose

by nearly six times at 11.5 mg/L, and both genes were clearly upregulated. In response to increasing oxidative stress, this increase implies that antioxidant defenses were transcriptionally activated at an early stage. This transcriptional upregulation at the moderate concentration likely represents an early adaptive regulatory response, indicating that the organisms were still capable of activating stress-responsive pathways under sublethal oxidative challenge. Other *Daphnia* species exposed to low or sublethal contaminant levels have shown comparable increases in antioxidant-related genes, where gene expression alterations take place as part of an early stress response (Poynton and Vulpe, 2009; Liu et al., 2022; Hu et al., 2023). However, both cat and sod expression decreased at the higher concentration (23 mg/L), which was in line with the decrease in enzyme activity. The decline in both transcripts and enzyme activity suggests that under more severe stress, regulatory efficiency was disrupted, possibly reflecting both stress-induced impairment and reduced protective capacity under prolonged exposure. Similar transcriptional suppression has also been reported in *Daphnia* (Lampert, 2011; Lee et al., 2024). The findings collectively imply that alterations in the expression of antioxidant genes may function as sensitive and early markers of oxidative stress caused by benzyl alcohol. In comparison with recent chronic exposure studies, both similarities and differences can be observed. De Felice et al. (2022) reported modulation of antioxidant-related genes, including sod and cat, during 21-day exposure of *Daphnia magna* to polystyrene nanoplastics. However, in their study, transcriptional changes were mainly influenced by exposure time, and treatment effects were generally limited. In contrast, the present results demonstrate a clearer concentration-dependent response, with marked upregulation at moderate exposure followed by suppression at higher stress levels. A comparable stress intensity-dependent regulation has also been described in *Daphnia pulex* exposed to chemical stressors (Liu et al., 2018), where disruption of antioxidant pathways occurred under intensified conditions. Together, these comparisons indicate that while antioxidant genes consistently respond to chronic stress, the magnitude and direction of regulation depend strongly on the nature and intensity of the contaminant.

4.4 Integrated mechanistic interpretation

When combining the results of the acute, enzymatic, and gene expression analyses, a distinct pattern of toxicity in *D. pulex* is revealed. The animals increased gene expression and enzyme activity, demonstrating that they could still activate their antioxidant defenses at moderate concentrations. This points to a transient adaptive reaction to oxidative stress. Yet, this protective ability decreased at greater concentrations. Gene expression, glutathione levels, and antioxidant enzymes all declined. This suggests that the antioxidant defense system was altered under prolonged stress, possibly reflecting an adaptive regulatory response at first, but with evidence of reduced protective capacity at higher exposure levels. Notably, these molecular and biochemical alterations occurred at concentrations significantly lower than the acute LC_{50}, demonstrating that physiological disruption starts long before mortality is apparent. Additionally, prior research has linked extended oxidative stress to decreased *Daphnia* growth, reproduction, and overall fitness, which could eventually affect population stability (Liess et al., 2016). Because of this, biochemical and molecular biomarkers are thought to be helpful in identifying sublethal toxicity before mortality-based endpoints are reached (Jemec Kokalj et al., 2023). The ecological relevance of the present findings is supported by the documented occurrence of benzyl alcohol in wastewater-related environments, indicating that aquatic organisms may encounter this compound under real-world exposure scenarios. Benzyl alcohol has been reported in wastewater treatment plant influents at ng/L levels, which confirms environmental occurrence even though these concentrations are lower than the experimental levels used here. Taken together with its demonstrated toxicity in aquatic models, these observations suggest that chronic low-level exposure may contribute to sublethal physiological disturbance in freshwater biota, with possible consequences for organismal fitness and ecosystem function (Musee et al., 2023; Manjunatha et al., 2021). Similar integrated biomarker-based approaches may therefore be useful for hazard assessment in *Daphnia* species. Overall, the findings of the present study indicate that oxidative stress is a major contributor to the long-term toxicity of benzyl alcohol.

5. CONCLUSION

In conclusion, benzyl alcohol caused measurable toxic effects in *D. pulex* under the tested exposure conditions. Although acute exposure resulted in only moderate mortality (48 h LC_{50}=229.68 mg/L), chronic exposure to much lower levels (11.5-23 mg/L; 5-10% of LC_{50}) produced significant biochemical and transcriptional disruptions. Together with glutathione depletion, the observed biphasic response of antioxidant enzymes and the marked induction of cat and sod expression (approximately 3-6-fold) suggest that oxidative stress is a major factor in this compound's toxicity. The significance of these sublethal biomarkers as early indicators of environmental stress is highlighted by the fact that they responded more quickly and sensitively than mortality. Overall, the enzymatic and molecular biomarkers supported their value as early-warning indicators of physiological stress because only two LC_{50}-derived sublethal concentrations were used, these responses should be interpreted as exposure-level-related patterns rather than a complete dose-response relationship. However, the molecular and biochemical biomarkers measured in this study should be interpreted as early mechanistic indicators of sublethal stress and potential precursors of higher-level biological effects, rather than as direct evidence of population-level impairment. Therefore, future studies should integrate these biomarkers with ecologically relevant apical endpoints, such as reproduction, growth, brood production, survival, and time to first brood, to better link benzyl alcohol-induced redox disruption with ecological outcomes. From an environmental perspective, the present findings suggest that benzyl alcohol may pose a risk to freshwater organisms even at nonlethal concentrations, supporting the value of sublethal biomarker responses as early-warning tools in aquatic environmental monitoring and risk assessment.

AUTHOR CONTRIBUTIONS

Ghufran Saad Aljanabi: Conceptualization, study design, experimental execution, sample collection, biochemical assays, molecular analysis (RT-qPCR), data curation, statistical analysis, visualization, and original draft preparation. Adel Mashaan Rabee: Methodological supervision, validation of experimental procedures, interpretation of results, critical revision of the manuscript for important intellectual content, and overall scientific guidance. All authors have read and approved the final version of the manuscript.

DECLARATION OF CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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