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RESEARCH

Gene expression responses unveil the negative effects of Olive Mill Wastewater on *Gammarus komareki* (Amphipoda: Gammaridae)

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Abstract
Olive Mill Wastewater (OMW) is a major organic pollutant, particularly in Mediterranean countries with intensive olive oil production. In this study, the toxic effects of OMW were tested on a freshwater macroinvertebrate, *Gammarus komareki*. Animals were exposed to different concentrations of 1/5, 1/10, and 1/20 of the previously calculated 72-h LC50 (LC50 = 3.65%) and was applied as high (0.73%), medium (0.365%) and low (0.1825%) levels, respectively for 20 days. At exposure, female and male individuals from each group were sampled separately. Samples were analyzed for the expression levels of antioxidant defences (*cat*, *gst*, *se-gpx*, and *mn-sod*), reproduction (vitellogenin-like protein, *vtg*), respiration (hemocyanin, *hc*), antitoxic defences (heat shock protein 70, *hsp70*), and molting (molt-inhibiting hormone, *mih*) related genes. Results revealed that the expression levels of *vtg* gene in females exposed to low doses were increased significantly compared to other groups; however, its levels were decreased in group exposed to high doses compared to the control and other groups. The mRNA expression levels of *mn-sod* and *se-gpx* genes of males and female individuals exposed to low OMW doses increased significantly compared to other doses. The mRNA expression levels of *gst* gene increased in males and females exposed to moderate doses and only in males exposed to low doses compared to other groups. The mRNA expression levels of *cat* gene of females were similar in all groups, while their levels were higher in males exposed to OMW than in the control group. The expression values of *hsp70* gene were significantly decreased in females and all males exposed to high doses of OMW. On the other hand, the *mih* gene expression in females was increased at low doses of OMW, while decreased in the groups exposed to high doses compared to the control group. In male individuals, the *mih* expression levels significantly increased in all experimental groups. The expression of the *hc* gene was decreased in male and female individuals exposed to all doses of OMW compared to the control group. To put it briefly, the great variations in the gene expression levels in groups exposed to OMW underlines the toxic effects of this effluent and its negative impacts on the aquatic organisms.

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Keywords Gammaridae · Crustacea · Olive Mill Water · Toxicity · Gene expression

Introduction

Mediterranean countries, including Türkiye, are major producers of olive oil (Tunalioglu et al. 2008). Olive mill wastewater (OMW) is generated during both one-phase and three-phase centrifugation processes, which involve removing soil and debris and malaxation to extract oil (Zahi et al. 2022). Its production rates range from 800 to 1200 L per ton of the processed olives (Tsagaraki et al. 2007), resulting in an annual discharge of approximately 30 million cubic meters (Cinar and Alma 2004). The olive oil production process consistently generates significant volumes of wastewater, which are increasing annually. Recent studies unveil that OMW is a significant environmental pollutant (Zahi et al. 2022). Although the quantity of OMW produced is relatively small compared to other industrial waste streams because of the seasonal nature of olive oil production, its impacts on the environment cannot be ignored (Kučić Grgić et al. 2018). According to its chemical nature, OMW is a complex acidic effluent (pH < 5.5) primarily composed of water (83–96%) with minor constituents like sugars, lipids, organic acids, pectin, and tannins (< 1.5% each) (Diamantis et al. 2013).

Previous studies have demonstrated the toxicity of OMW and its polyphenolic fractions to freshwater organisms, bacteria, and seeds (Paraskeva and Diamadopoulos 2006). Besides, the phenolic compounds in OMW pose significant health risks to communities living in contaminated areas. The detrimental impacts of OMW extend beyond marine ecosystems, posing significant challenges to freshwater aquaculture as well. In freshwater ecosystems, studies also have shown that OMW was often discharged directly into freshwater ecosystems without any treatment (Haddad et al. 2017). A research study demonstrated that exposure to OMW resulted in genotoxic and histopathological impacts on the tissues (gill, liver and muscle tissues) of one of the freshwater fish species, *Lepomis gibbosus* that were caught from a Turkish lake (Topçam dam lake) (Koca and Koca 2016). In marine environment, previous research has demonstrated that OMW induces toxicity in the marine environment (Danellakis et al. 2011), which can negatively impact the health status and survival of aquaculture species. Those authors explained that OMW has a potential ability to induce genotoxic, cytotoxic, neurotoxic, and oxidative effects in mussels, *Mytilus galloprovincialis* tissues (Danellakis et al. 2011). In addition, the detrimental effects of OMW have been demonstrated on various aquatic organisms, including crustaceans and insects. For instance, exposure to OMW has been shown to negatively impact reproduction in species like *Daphnia longispina*, *Thamnocephalus platyurus*, and *Heterocypris incongruens* (Michael et al. 2014). Additionally, high concentrations of OMW (4 ml/liter) can lead to high mortality rates in other species, such as *Gammarus pulex* within 24 h of exposure (Karaouzas et al. 2011). Therefore, recent research highlights the critical importance of managing and treating OMW to mitigate its environmental damage. To this end, treating or significantly reducing OMW waste has become a pressing concern (Cabrera et al. 1996; Fiorentino et al. 2003).

The family *Gammaridae* exhibits a broad distribution, predominantly concentrated in Europe and extending into Asia. Within this family, the genus Gammarus Fabricius, 1775 stands out as one of the largest genera of Amphipoda, encompassing over 200 described species to date (Väinölä et al. 2008). Gammarids constitute a primary food source for fish (especially trout) and several other freshwater fish species (MacNeil et al.

2000; Graeve et al. 2001). Gammarus is represented by 34 taxa in the basins of Türkiye (Özbek and Ustaoğlu 2006). The amphipod *Gammarus komareki* exhibits a widespread distribution particularly in Bulgaria, Greece, Iraq, and Türkiye (Grabowski and Pešic 2007; Zamanpoore et al. 2011). Gammarus species are widely distributed in the aquatic systems and gain special importance because of their roles as effective bioindicators due to their ability to efficiently accumulate heavy metals (MacNeil et al. 1997; Gerhardt et al. 2007). There are only few studies about toxic effects of OMW have been carried out with these organisms. Thus, the objective of this study was to assess the acute and chronic toxicity of OMW on *G. komareki* at morphological, behavioral and molecular levels. Additionally, its impact on the hormone levels of male and female individuals have also been assessed as a part of the study.

Material and methods

Animals

G. komareki utilized in this study were collected using a hand net from the Karamenderes River (39°53′12.2″N, 26°15′28.5″E) located in the Northern Aegean Basin of Çanakkale Province, Türkiye. Their live population was transported to the laboratory of the Faculty of Marine Sciences and Technology at Çanakkale Onsekiz Mart University. In the laboratory, they were acclimated in 40-L transport containers for one week prior to the experimentation.

OMW processing

The OMW used in the experiment was sampled during the olive oil production season from a private olive oil facility operating in Çanakkale Province, Türkiye. Upon arrival at the laboratory, several water quality parameters of the OMW were measured using a HANNA HI98194 Multi-Parameter Meter, which analyzes pH, ORP, EC, TDS, dissolved oxygen (DO), pressure, and temperature (according to Table 1). Subsequently, a portion of the OMW was stored in a refrigerator at 4°C, while the remaining product was preserved at −20°C in the Laboratory's freezer, following the experimental procedures.

Acute toxicity experiment (LC50 testing)

Before the experimentation, specimens were left to be acclimated to laboratory conditions for 7 days. The water temperature was precisely controlled, and continuous aeration was maintained using an aquarium-type air pump. In the custom-designed experimental unit and throughout the experiments, the organisms were subjected to a photoperiod program of 12h:12h light:dark cycle. The experimental setup consisted of six groups, including a

Table 1 Some physico-chemical properties of OMW used in the experiment

pH value	mVpH	mVORP	Conductivity (mS/cm)	mS/cm[a]	Total Dissolved Solids (TDS; mg/L)	Temperature (C°)
4.9	132	−5.5	10000,46	12,000,34	5678	35

control group, with each group containing 20 test specimens. The LC50 value (3.65%) was initially reported in a conference proceeding (Odabaşı et al. 2021), and in this study, a full experimental validation was conducted under controlled conditions to confirm the accuracy and applicability of the previous findings. Upon exposure of the specified doses, the 72-h acute experiment commenced and LC50 was calculated using SPSS probit analysis. Throughout the experiment, pH, temperature, and electrical conductivity were measured and recorded using a multiparameter device (Hanna, HI98194).

Chronic toxicity experiment

To investigate the effects of different OMW concentrations on *G. komareki*, 12 small glass aquariums with dimensions of 13.5-cm length, 12.5-cm width and 14.5-cm height and a total volume of 2.4 L were used. Each aquarium was filled with 1.5 L of clean dechlorinated tap water. To evaluate the chronic effects of OMW, 1/5, 1/10, and 1/20 of the 72-h LC50 value of 3.65% (Odabaşı et al. 2021) was applied as high—0.73%, medium—0.365% and low—0.1825% levels, respectively for 20 days. Each concentration (0, 0.73, 0.365, and 0.185%) was tested in triplicate, with 20 organisms in each replicate, resulting in a total of 60 organisms per tested group. *G. komareki* fed ad libitum with decomposed oak (*Quercus* spp.) leaves during the chronic toxicity experiment. As described in the acute experiment, pH, temperature, and electrical conductivity values were monitored daily using a multiparameter instrument (Hanna, HI98194) in all test groups. Moreover, the activities of the organisms were monitored day by day.

Morphological alterations and behavioral responses

The behavioral responses of *G. komareki* individuals were assessed through direct manual observations without using recording devices. After acclimation, individuals were observed in the experimental setup, and their behaviors were systematically recorded daily based on predefined criteria. In order to gain insights into the morphological alterations and behavioral responses of animals subjected to different doses of OMW in both acute and chronic experiments, animals were evaluated based on their feeding, horizontal and vertical movement tendencies, tendency to close to surface of the bottom, locomotion speed and darkening in body appearance, sinking tendencies, locomotion speeds, and physical appearance, rated on a scale from 1 (worst) to 10 (best). Behavioral dynamics have been rated based on a long-term observation of each individual. The effects of toxicity levels on the organisms were observed accordingly. The control group was assigned a score of 10 for all dynamics. Observations were conducted following a standardized protocol, and data were manually documented for subsequent analysis. Behavioral differences between control and treatment groups were evaluated by comparing the recorded parameters. This approach ensured a consistent and reliable assessment of the behavioral effects of OMW exposure on *G. komareki*.

Sampling procedures

At the end of the 20-day chronic experiment, the whole body of female and male individuals from each test group were sampled separately. These samples were preserved in RNA later solution and sent to an external service provider for analysis of the gene expression levels.

Gene expression analyses

Female and male *G. komareki* individuals (six individuals/group) were sampled and preserved in RNA later solution. The total RNA was extracted from whole-body samples using the Thermo Scientific GeneJET RNA Purification Kit (Waltham, Massachusetts, USA) in line with the manufacturer's protocol. Approximately 30 mg of the whole-body tissue was used for RNA isolation. The samples were homogenized in 300 µL of lysis buffer containing β-mercaptoethanol. Following the manufacturer's guidelines, the total RNA was isolated through centrifugation. The concentration and purity of the RNA were measured with a Multiskan GO spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA). RNA samples were diluted to a final concentration of 15 ng/µL and stored at − 80 °C until cDNA synthesis.

The cDNA synthesis was carried out using the OneScript® Plus cDNA Synthesis Kit (Applied Biological Materials Inc. (abm), Richmond, Canada). The reaction mixture consisted of 1 µg of RNA, 1 µL of oligo dT primer (10 µM), 4 µL of 5 × RT Buffer, 1 µL of OneScript® Plus RTase (abm), and nuclease-free water, bringing the final volume to 20 µL. The reaction was incubated at 50–55 °C for 15 min using a thermal cycler (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The resulting cDNA was quantified and assessed for purity using the Multiskan GO spectrophotometer. Samples were adjusted to a 20 ng/µL concentration and stored at − 20 °C until used for gene expression analysis.

The qRT-PCR was performed on the StepOnePlus TM Real-Time PCR system (Applied Biosystems, Waltham, Massachusetts, USA) with the 5 × HOT FIREPol EvaGreen HRM Mix (Solis BioDyne, Tartu, Estonia). Specific primers for target genes were used, as detailed in Table 2. The thermocycling conditions for RT-qPCR were as follows: an initial denaturation at 95 °C for 300 s, followed by 15 s at 95 °C, then annealing and extension at 55–68 °C for 60 s. Data analysis was performed using the 7500 System SDS software (version 1.3.1, Applied Biosystems). The gene expression levels were normalized to the reference gene *β-actin*, and the relative expression was calculated using the $2-^{\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistical analysis

Before conducting statistical analysis, Kolmogorov–Smirnov and Levene's tests were used to check data normality and variance homogeneity. Subsequently, one-way ANOVA was applied to evaluate differences between the test groups. Mean differences were tested at a 5% significance level using Duncan's multiple range test as a post-hoc analysis. Probit analysis and other statistical evaluations were performed using SPSS 19 (IBMM SPSS Statistics 19).

Results

Results of the acute toxicity study

The Probit Analysis method evaluated the acute toxicity of OMW on *G. komareki*. The calculated 72-h LC50 value was determined as 3.65% (as presented in Fig. 1). The regression equation for the probit model was $y = 11.014x - 1.2084$, and the coefficient of determination was $r^2 = 0.89$ ($p < 0.05$), indicating that there was a strong correlation between the logarithmically transformed OMW concentrations and the probability of death. No deaths were recorded at the lowest concentrations tested (2.33% and below). However, as

Table 2 Primer sequences of *Gammarus* genes used in the present study

Genes		Primers		NCBI GenBank accession numbers (References)
Catalase	*cat*	Forward	CATCCACACACAGAAACGCAAG	(de Melo et al. 2021)
		Reverse	CTCGGTCCGTAAACAGGAAG	
Hemocyanin	*hc*	Forward	AAAGGATGAACGAGGGCGAG	(de Melo et al. 2021)
		Reverse	GGAGTTGGTGAACAAGTGTGG	
Glutathione S-transferase	*gst*	Forward	CATGGCGCCTATTCTTGCTT	LT575461.1
		Reverse	GCAAGACTTGTCATAGGCCG	
Manganese superoxide dismutase	*mn-sod*	Forward	CGCCTACTACCTGCAGTACA	LT575462.1
		Reverse	AAAACGCTCAGCCAAACCAA	
Vitellogenin-like protein	*vtg*	Forward	GTCTGGACATGAGCCCGATA	LT575464.1
		Reverse	GAAGAGGCATCGGGTGGG	
Heat shock protein 70	*hsp70*	Forward	CTCTTCAACCCAGGCCAGTA	JN704340.1
		Reverse	CTTGTCGAGCTTGGCATCTC	
Molt-inhibiting hormone	*mih*	Forward	CTTCCGCTCGTCAAAGATCG	MF405139.1
		Reverse	TCTTCCTCATGTACTCCGGC	
Selenium-dependent glutathione peroxidase	*se-gpx*	Forward	GCTTCCCGACCAATCAGTTT	LT575463.1
		Reverse	TCTGAAACATGTCCCCGAGA	
Beta actin	*β-actin*	Forward	TGGCCGTGACCTTACAGATT	LT575466.1
		Reverse	AGTTGTAGGTGGTCTCGTGG	

the exposure concentration increased to 2.67% and 3.00%, the mortality rates increased to 6.67% and 11.11%, respectively. A sharp increase in mortality was observed at the 3.33% dose, occurring at 47.78%, while the highest concentration resulted in a mortality of 61.11%.

Morphological alterations and behavioral responses

In the acute experiment, the digestive system of individuals exhibited a dark appearance following exposure. Moreover, there is a slowdown in the movements of individuals at the lowest doses (2.3% and 2.67%) compared to individuals in the control unit. Additionally, while the individuals present in the control and low-dose groups were distributed in the bottom region of the test unit, individuals exposed to a moderate level (3%) of OMW tended to gather in the middle regions of the test unit. At the highest doses (3.33% and 4%), there is a tendency of animals to gather in the surface region and exhibited vertical movement. After the control group, a significant decrease was observed in individuals' preference for the surface area of water. However, individuals in the medium-dose group exhibited a distribution in the bottom of the experimental unit, unlike the low and high-dose groups. The feeding behavior of individuals showed a significant change at medium (3.33%) and high (4%) doses compared to low doses. It is believed that this effect is due to the stress induced by higher doses, which adversely affected their feeding behavior.

In the chronic toxicity experiment, individuals at the low dose did not exhibit behavior different from the control group. The individuals at the moderate and high dose showed a

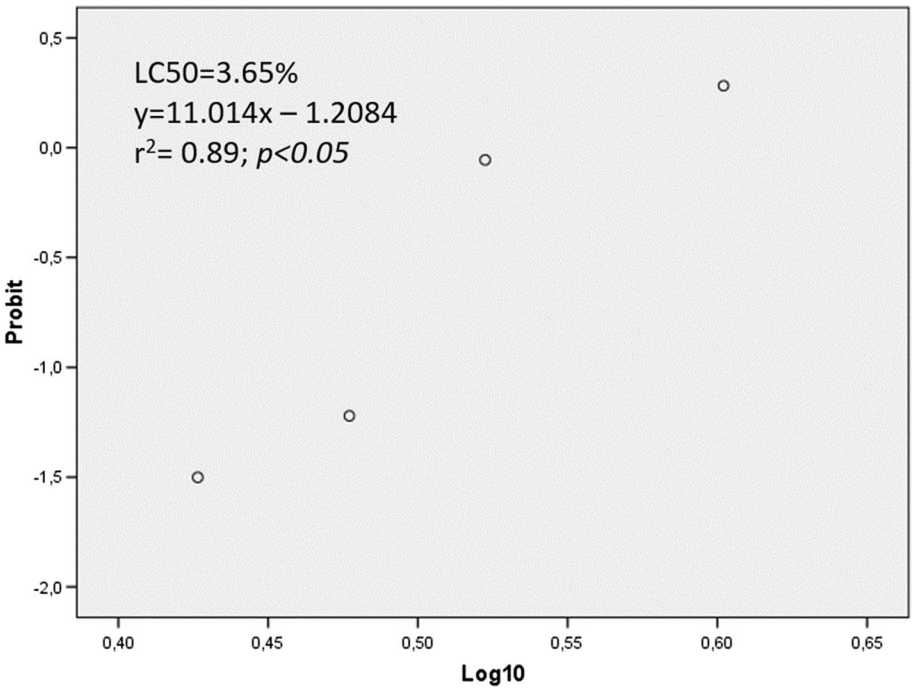


Fig. 1 Probit graphic method for estimation of LC50 value of different OMW doses on *G. komareki*

slowdown in movements compared to the control group. It was seen that the control, low, and moderate dose groups preferred the bottom regions. The individuals at the high dose did not demonstrate observational differences in movement compared to individuals at the moderate dose. However, these individuals were observed to take positions closer to the surface compared to all other groups. The digestive system of individuals exhibited a dark appearance following exposure. The survival was recorded throughout the chronic toxicity experiment, and all individuals remained alive in all treatment groups.

Gene expression responses

The results of the gene expression levels of female and male individuals exposed to low, medium, and high concentrations of OMW for 20 days are presented in Fig. 2 and Fig. 3, respectively. Results revealed that the expression levels of the reproduction-related vitellogenin (*vtg*) gene in female individuals significantly increased at low doses ($p < 0.05$), while at higher doses, their levels decreased progressively, showing a significant reduction compared to the control and low dose groups ($p < 0.05$).

When examining the antioxidant defense-related gene responses, both female and male individuals showed significant increases in the expression of *mn-sod* and *se-gpx* genes in the low-dose OMW exposure ($p < 0.05$). The expression of *gst* gene increased in female individuals exposed to medium doses of OMW and in male individuals exposed to low and medium doses compared to other groups ($p < 0.05$). The *cat* gene expression level was found to be similar across all experimental groups in females, while male individuals exposed to low and medium OMW doses showed significantly higher *cat* gene expression levels compared to the control group ($p < 0.05$).

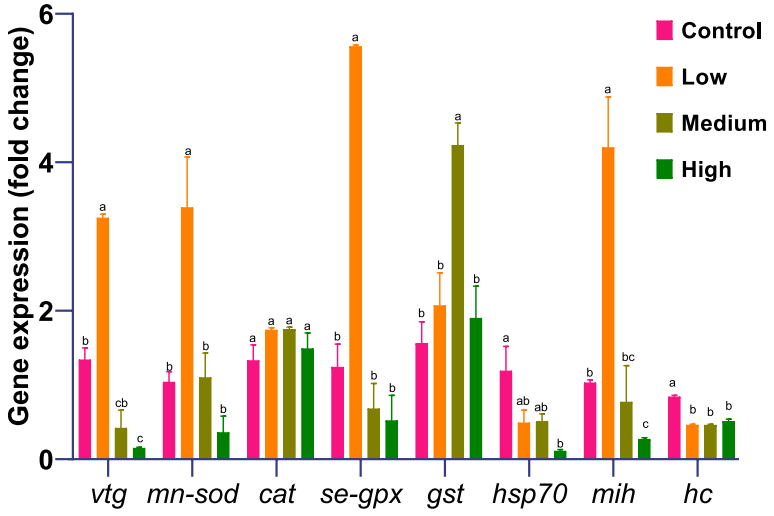


Fig. 2 Gene expression responses of female *G. komareki* individuals ($n = 6$ per group) after chronic exposure to different doses of Olive Mill Wastewater. Experimental groups represented by different letters indicate statistically significant differences ($p < 0.05$). Error bars represent the standard error of the mean (SEM)

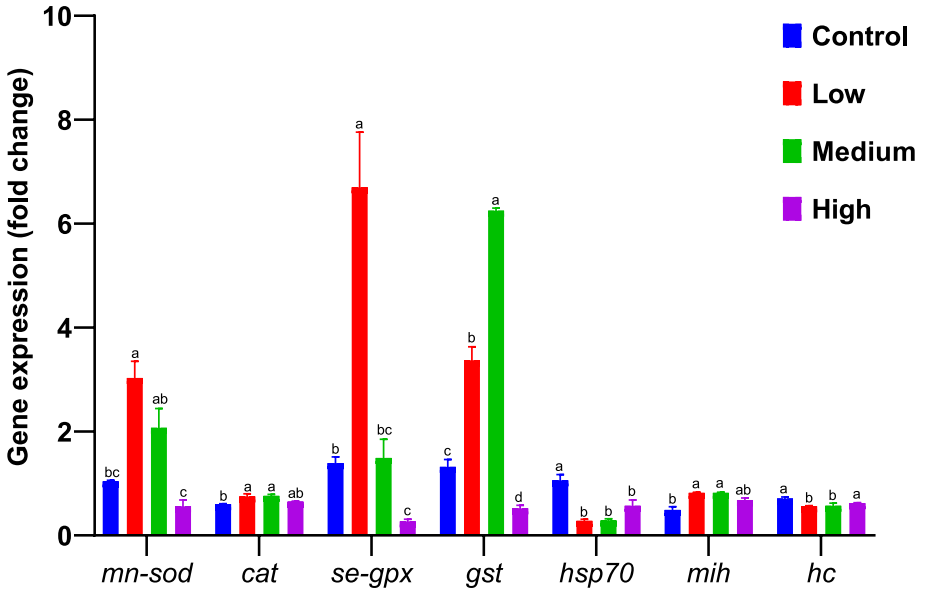


Fig. 3 Gene expression responses of male *G. komareki* individuals ($n=6$ per group) after chronic exposure to different doses of Olive Mill Wastewater. Experimental groups represented by different letters indicate statistically significant differences ($p<0.05$). Error bars represent the standard error of the mean (SEM)

Regarding the antitoxic defense-related *hsp70* gene levels, a significant decrease was observed in female individuals exposed to high doses of OMW and in all male individuals in the experimental groups exposed to OMW ($p<0.05$). The *mih* gene associated with the endocrine system showed an increase in female individuals at low doses but a decrease at high doses compared to the control. In low and medium doses exposure male individuals in the experimental groups, a significant increase of *mih* gene expression levels was noted ($p<0.05$). For the respiratory system, the *hc* gene expression, was found to decrease in both female and male individuals across all OMW exposure groups (except high doses male individuals) compared to the control group ($p<0.05$).

Discussion

This study investigates the acute and chronic toxic effects of OMW on *G. komareki*. The acute toxicity results showed a dose-dependent increase in mortality with an LC50 value of 3.65%. These findings highlight the potential risks of blackwater pollution in freshwater ecosystems, particularly in areas where high concentrations may accumulate due to industrial discharge. In addition to acute toxicity effects, we examined changes in reproductive responses, antioxidant defense, antitoxic defense, and endocrine and respiratory system-related gene responses in females exposed to OMW at chronic concentrations in both male and female *G. komareki*.

In most oviparous organisms, the vitellogenin (Vtg) and vitellogenin-like proteins are precursor molecules of yolk proteins (i.e., vitellin), which provide the sole energy reserves during the embryonic development and thus play a crucial role in reproduction

(Xuereb et al. 2011). In the present study, it was found that the *vtg* gene expression levels were elevated in sexually mature females and increase further during gametogenesis, whereas the *vtg* gene is weakly expressed in juveniles and at very low levels in males (Xuereb et al. 2011). In addition, it was found that low exposure doses of OMW resulted in the increase of *vtg* gene expression levels in female individuals, while high doses have the opposite effect. This phenomenon is thought to be related to the phytoestrogens present in OMW (Karen and Frederick 2024). Research elucidated that the main phytoestrogen compounds found in OMW were identified as lignans, such as (+)-pinoresinol and (+)−1-acetoxy pinoresinol (Brenes et al. 2000; Owen et al. 2000). Pinoresinol has a chemical structure similar to estrogen, which allows it to interact with estrogen receptors (ERα) (Lopez-Biedma et al. 2016). Previous study has shown that lignans such as pinoresinol can up-regulate *pS2* gene expression by activating estrogen receptor pathways via estrogen response elements (ERE) (Wang et al. 2011). These findings suggest that phytoestrogens in OMW, including pinoresinol, may similarly modulate other estrogen-responsive genes, such as *Vtg*, in aquatic organisms. This study's findings show the need for further experimental investigations into the reproductive effects of OMW on aquatic crustaceans, mainly focusing on estrogenic pathways and their potential disruption.

SOD and CAT are known as the main enzymes capable of detoxifying reactive oxygen species (ROS). The dismutation of the superoxide anion (O_2^-) into the less reactive hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) is catalyzed by SOD and is among the primary antioxidant defense mechanisms against oxidative stress (Fridovich 1995). GPX enzyme plays a significant role in inhibiting the lipid peroxidation process and thus protects cells from oxidative stress (Ighodaro and Akinloye 2018). In this study, it was observed that the expression levels of *mn-sod* and *se-gpx* genes in both male and female individuals significantly increased with low-dose OMW exposure. At higher doses, the gene expression levels were similar to the control, suggesting that the increases in expression levels may vary depending on the dose and duration of exposure.

The *cat* gene expression levels were significantly higher in male individuals exposed to low and medium doses of OMW compared to the control group. CAT is a type of ROS-scavenging enzyme that decomposes H_2O_2 into O_2 and H_2O, thereby removing H_2O_2 from the body. Consequently, CAT is considered a fundamental enzyme for the biological defense system of living organisms. Our research findings align with a previous study involving Gammarus individuals exposed to 2,2′,4,4′-tetrabromodiphenyl ether (Gismondi et al. 2015), highlighting sex-specific differences in antioxidant responses in *G. komareki* exposed to OMW. The significantly higher *cat* gene expression observed in males compared to females following OMW exposure suggests a stronger antioxidant activation in males under oxidative stress conditions. The absence of similar results in female individuals may be attributed to differences in molting stages and the varying impacts of environmental pollutants on gene levels at different times due to the egg production cycles of females. Furthermore, the elevated expression of chaperone proteins in female Gammarus individuals, as reported by Gismondi et al. (2015), may contribute to females' enhanced resistance to protein damage and a more stable oxidative balance. Similarly, Sroda and Cossu-Leguille (2011) demonstrated that the *cat* gene was overexpressed in males of *Gammarus roeseli* and *Dikerogammarus villosus* exposed to copper, whereas females exhibited under expression.

GST is induced under oxidative stress conditions and plays a role in detoxifying organic epoxides, hydroperoxides, and unsaturated aldehydes formed after lipid peroxidation, by conjugating these products with GSH (Fernández-Iglesias et al. 2014). The *gst* gene expression increased in the group of female individuals exposed to medium

doses of OMW and in the groups of male individuals exposed to low and medium doses of OMW compared to other groups.

The elevation in *mih* gene expression levels observed in both male and female *G. komareki* individuals suggests that exposure to OMW adversely impacts their development. The increase in *mih* gene expression levels observed in both male and female *G. komareki* individuals suggests that OMW exposure negatively affects their development. This upregulation may be linked to a disruption in the molt cycle, since molt inhibitory hormone plays an important role in regulating ecdysteroid hormone secretion (Hyne 2011). Previous studies have reported that long-term exposure to pollutants such as heavy metals may trigger feedback mechanisms, leading to increased *mih* expression, possibly as a compensatory response to premature ecdysteroid synthesis induction (Gismondi 2018). In addition, delayed molt has been associated with a decrease in growth and reproductive success in crustaceans (Weis 1978; de Melo et al. 2021). These findings suggest that OMW exposure may interfere with the endocrine pathways controlling molt, potentially affecting the fitness and survival of *G. komareki* populations.

Hemocyanin is a copper-containing respiratory protein that plays a critical role in oxygen transport for arthropods (Hagner-Holler et al. 2005). Furthermore, the observed reduction in *hc* gene expression, a crucial protein for oxygen transport in arthropods and crustaceans, across all OMW-exposed groups compared to the control group, indicates a potential decrease in oxygen consumption. To date, no studies have explored the molecular-level effects of OMW on gene expression in Gammarus species. However, existing literature highlights the high toxicity of OMW and its polar fractions to a wide range of organisms, including microorganisms, plants, invertebrates, and vertebrates (Babić et al. 2019). Previous studies have shown that *hc* gene expression increases with increased oxygen demand in Gammarus individuals exposed to heat stress (Lipaeva et al. 2023). On the other hand, de Melo et al. (2021) investigated the effect of mercury exposure in *Gammarus fossarum* at two different temperatures and revealed that heat stress increased hemocyanin protein levels but did not change the mRNA gene expression levels, suggesting that hemocyanin protein can be regulated to increase oxygen carrying capacity, but this may not always occur at the transcriptional level. This study observed decreased *hc* gene expression in *G. komareki* individuals exposed to OMW. This decrease may be related to metabolic depression and decreased oxygen consumption caused by toxic stress. The decreased mobility observed in individuals exposed to OMW may indicate decreased metabolic activity and *hc* gene expression.

Conclusions

This study unveils that OMW induces oxidative stress in *G. komareki* individuals, with the severity varying based on dose, exposure duration, and sex. Further research is needed to fully understand the long-term effects of OMW-induced oxidative stress on male and female *G. komareki* specimens. In addition, the phytoestrogens present in OMW may play a role in disruption of the reproductive performance of *G. komareki.* The findings of this research also highlight the importance of managing and treating OMW to mitigate the environmental damage and their toxic effects on aquatic organisms.

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Author contributions Sevdan Yilmaz: Conceptualization; Writing—original draft; Investigation; Methodology; Validation; Formal analysis, Deniz Anıl Odabaşı: Methodology; Data curation; Validation; Formal analysis, Aytuğ Zilifli: Writing—original draft; Data curation; Methodology; Validation; Formal analysis, Mohammed Banni: Data curation; Methodology; Formal analysis, Hany M.R. Abdel-Latif: Writing—original draft; Data curation; Writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Ethics approval The amphipod Gammarus, being a crustacean, is currently not subject to regulatory requirements for its experimental manipulations in the laboratory. Despite this, we took care to comply with the 3R rule. We used as few Gammarus individuals as possible in our experiments. Additionally, we anesthetized sacrificed Gammarus individuals on ice before slaughtering or euthanizing them. For euthanasia, we kept it on ice until it was completely inactive.

Consent to participate Not applicable.

Competing interest The authors declare no competing interests.

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