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# Copper-Induced Ionoregulatory Disturbance, Histopathology, and Transcriptome Responses in Freshwater Mussel (*Anodonta woodiana*) Gills

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**Abstract:** Copper (Cu) contamination has become a severe problem in freshwater environments worldwide. The freshwater mussel *Anodonta woodiana* is used as a unique bioindicator to monitor Cu contamination in freshwater environments. However, Cu toxicity and response mechanisms in *A. woodiana* are still largely unknown. A sublethal acute exposure experiment (2.0 mg/L Cu exposure for 72 h) was conducted to investigate the effects of Cu bioaccumulation on ionoregulatory homeostasis, histological features, and transcriptome responses using *A. woodiana* gills as indicator tissue. The gill bioaccumulation capacity was up to 474. Cu bioaccumulation decreased $Na^+$ and $Mg^{2+}$ concentrations ($p < 0.05$) by 82% and 17%, respectively, and induced cilia loss, epithelial desquamation, and filament atrophy of the gills. Transcriptome analysis identified 3160 differentially expressed genes (DEGs), including 1870 upregulated and 1290 downregulated genes. GO enrichment analysis showed that cellular processes, metabolic processes, biological regulation, and responses to stimuli contained the most DEGs in the biological processes. KEGG pathway analysis showed that apoptosis, arginine and proline metabolism, the toll-like receptor signaling pathway, apoptosis-multiple species, histidine metabolism, beta-alanine metabolism, cytokine–cytokine receptor interaction, and the p53 signaling pathway were significantly enriched. These findings provide comprehensive evidence for exploring Cu toxicity and response mechanisms in freshwater mussels.

**Keywords:** aqueous Cu; freshwater mussel; bioaccumulation; ionoregulatory; histopathology; transcriptome

**Key Contribution:** This study reveals the toxicity mechanism of Cu overaccumulation in freshwater mussel *A. woodiana* in terms of ionoregulatory disturbance, histopathology, and transcriptome responses.

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## 1. Introduction

Copper (Cu) contamination has become a severe problem in freshwater environments worldwide owing to industrial emissions, mining discharge, agricultural activities, and fishery use (especially the use of copper sulfate ($CuSO_4$) as an algicide and fungicide) [1,2]. For example, Cu concentrations in surface water could elevate to 1.2–23.8 mg/L in many countries, such as in the USA [3], China [4], Spain [5], and Nigeria [6], severely threatening ecological safety and human health. Cu is an essential metal that is important for numerous physiological processes in aquatic organisms, being a cofactor for oxidation/reduction reaction enzymes [7–9]. Cu is also involved in oxygen transport in the hemolymph as a component of hemocyanin in mussels [7,8]. However, elevated concentrations in water can be toxic by disrupting normal metabolic processes [7–9]. Notably, Cu is more toxic

than other metals (Hg, Cd, Zn, Pb, and Cr) to freshwater organisms [10]. Invertebrates are generally more sensitive to Cu than vertebrates [10]. Freshwater mussels have been experiencing a serious population decline globally [11,12]. Cu stress remains one of the key factors contributing to their decline in many regions [11,12]. However, the toxic mechanisms of Cu in freshwater mussels are still not well understood. The lack of gene sequences of most freshwater mussels [13] is one of the important reasons. In recent years, high-throughput RNA sequencing (RNA-seq) without a reference genome sequence has enabled the transcriptome analysis of freshwater mussels exposed to Cu and will hopefully uncover its molecular mechanisms of toxicity [14].

The freshwater mussel *Anodonta woodiana*, commonly known as the Chinese pond mussel, is native to the Yangtze and Heilongjiang Rivers of China [15]; however, it has spread across Asia (Indonesia, the Philippines, Turkey, Malaysia, Vietnam, Singapore, Uzbekistan, South Korea, Laos, and Kazakhstan), Europe (Croatia, Montenegro, Russia, Hungary, France, Romania, Italy, Germany, Austria, Slovakia, Czech Republic, Poland, Serbia, Moldova, Ukraine, Sweden, the Netherlands, Belgium, Slovenia, and Bulgaria), and the Americas (Costa Rica, Dominica, America, and Haiti) over the past few decades [16]. This mussel plays an important role as an aquatic ecosystem engineer [16], a food source [17,18], and in the pearl culture industry [19]. Moreover, it has been successfully used as a unique bioindicator in the "Freshwater Mussel Watch" project [20–22]. To date, many countries (e.g., China, Poland, Serbia, Bulgaria) have utilized *A. woodiana* to biomonitor Cu contamination in freshwater environments [21–25]. However, Cu toxicity to *A. woodiana* has not been explored in addition to reporting the changes in their protein content [26] and antioxidant enzymes [27].

Among all the organs of mussels, gills are one of the focal points in toxicological studies. Mussel gills are vital organs involved in respiration and filtration [28,29]. They are able to accumulate higher concentrations of Cu than other organs [29–31] because of their high surface ratio and direct water contact with a high throughput of volume. Therefore, mussel gills have been proposed as an indicator tissue for studying metal toxicity [29,32].

Our previous study showed that the 96 h $LC_{50}$ of Cu was 3.4 mg/L (95% confidence interval: 2.1–6.5 mg/L) for *A. woodiana* juveniles [27]. In this study, *A. woodiana* juveniles were exposed to 2.0 mg/L Cu (approximately 1/2 of the 96 h $LC_{50}$) for 72 h to reveal the mechanism of Cu toxicity to the mussels in terms of ionoregulatory homeostasis (especially $Na^+$, $Mg^{2+}$, $K^+$, and $Ca^{2+}$), histological features, and transcriptome responses using the gills as indicator tissue. This sublethal acute exposure could reveal Cu toxic effects on mussels and avoid mussels' adaptation to Cu exposure. We hypothesized that excessive Cu accumulation could cause ion loss, histological hazards, and disrupt gene expression. These results provide new insights into Cu toxicity mechanisms in freshwater mussels.

## 2. Materials and Methods

### *2.1. Mussels and Treatment*

Juveniles of *A. woodiana* of similar sizes (shell lengths of 5.5 ± 0.3 cm and body weights (including shells and soft tissues) of 21.1 ± 2.0 g) were obtained from the Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences (Wuxi, China). After removing the epibionts, the mussels were acclimatized in American Society of Testing Materials (ASTM) reconstituted soft water [33] (pH 7.3–7.5, hardness 40–48 mg/L, alkalinity 30–35 mg/L as $CaCO_3$) at 20 ± 1 °C with a 16:8 h light and dark photoperiod for 2 weeks. Cu exposure was carried out according to the ASTM standard guide for conducting laboratory toxicity tests with freshwater mussels [33]. Thirty mussels were randomly divided into control and Cu-treated groups using three replicate tanks (2.5 L solution per tank) with five mussels each. Based on the filtration rate (260 L/h/kg dry weight (d.w.) of soft tissue) of *A. woodiana* [34], it was estimated that it took approximately 7 h for the solution to be filtered by the mussels. The solution was continuously aerated during the experiment, and dissolved oxygen was higher than 7 mg/L. Cu was not detected (the detection limit is 0.3 μg/L) in the water of the control group. Mussels in the Cu-treated group were

exposed to nominal 2.0 mg/L Cu in the form of $CuSO_4 \cdot 5H_2O$ (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) for 72 h. The mean measured concentration of Cu was 2.05 ± 0.1 mg/L. The Cu concentration used in this study was based on the sublethal concentration limit (1/2 of the 96 h $LC_{50}$ of aqueous Cu) to *A. woodiana* juveniles [27]. Although this is a concentration characteristic of heavily contaminated natural waters and one that is not that often encountered in the environment, it may be suitable for investigating the mechanisms of acute toxicity. The short 72 h exposure could prevent mussels from adapting to Cu toxicity [30]. The test solutions were 100% renewed every 24 h to maintain Cu concentration stability and to reduce nitrogenous waste.

### *2.2. Copper Bioaccumulation and Ion Concentration Analyses*

After 72 h of Cu exposure, three mussels from each group were randomly chosen, and their gills were dissected using stainless steel scalpels. The concentrations of total Cu and key ions ($Na^+$, $Mg^{2+}$, $K^+$, and $Ca^{2+}$) in the gills were determined according to established protocols with slight modifications [22,35]. Briefly, each sample was dried to a constant weight for 24 h at 80 °C, digested in $HNO_3$ (Merck, 65%) with an ETHOS A T260 microwave digestion system (Milestone Inc., Milan, Italy), and analyzed using an Agilent 7500ce ICP-MS (Agilent Technologies, Tokyo, Japan). Concurrent measurements of certified reference material (DOLT-5 dogfish liver; National Research Council of Canada, Ottawa, ON, Canada) indicated that the recoveries of Cu and major ions ranged from 97% to 107%. Concentrations of Cu and ions in the gills were determined on a d.w. basis.

### *2.3. Histopathologic Analysis*

Three mussels from each group were randomly selected for histopathological examination. Two transverse tissue sections and two longitudinal sections were prepared from each individual [36]. Gills were collected, and the samples were fixed using Bouin's solution. The samples were subjected to alcohol gradation (70%, 80%, 90%, 95%, and 100%) before being embedded in paraffin blocks. Transverse sections of the gills and their longitudinal sections (5 μm thick) were cut and stained using hematoxylin and eosin. An Olympus BX51 microscope (Olympus, Tokyo, Japan) was used for observation and tissue photomicrography.

### *2.4. RNA Isolation, Library Construction, and Sequencing*

Three mussels from each group were used for transcriptome analysis. Total RNA was extracted from gills using the RNAiso Plus Reagent (TaKaRa, Shiga, Japan) according to the manufacturer's protocol. The RNA integrity and quantity of each sample were determined using an Agilent 2100 Bioanalyzer (Agilent, Shanghai, China) and a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Poly(A)+ mRNA was isolated using oligo (dT) beads. The mRNA was randomly interrupted by adding a fragmentation buffer. Single-stranded cDNA was synthesized using random hexamers, and double-stranded cDNA was synthesized by adding buffer, dNTPs, and DNA polymerase I, followed by the purification of double-stranded cDNA using AMPure XP beads. Purified double-stranded cDNA was end-repaired, A-tailed, and ligated to the sequencing linker. AMPure XP beads were used to control fragment size, and polymerase chain reaction (PCR) enrichment was performed to obtain a cDNA library.

After library construction, the quantification and insert size of the library were detected using a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and an Agilent 2100 Bioanalyzer instrument (Agilent Technologies, Santa Clara, CA, USA), respectively. The effective concentration of the library was accurately quantified by real-time quantitative RT-PCR (qRT-PCR) to ensure library quality. The cDNA sample was sequenced using the Illumina NovaSeq 6000 strategy with 150 bp paired-end reads. The RNA-seq datasets were deposited in the National Center for Biotechnology Information (NCBI) database under the accession number SRR15677615-SRR15677620 in BioProject PRJNA759042.

### 2.5. Transcriptome Data Analysis and Identification of Differentially Expressed Genes

Raw data in Fastq format were processed using the Illumina sequencing platform. Adaptor, poly-N, and low-quality sequences were removed from the raw data to obtain clean reads. The Q20, Q30, and GC content and sequence duplication levels of clean reads were calculated. De novo transcriptome (without a reference genome) assembly was performed using Trinity software (v2.4.0) [37].

Bowtie and RNA-seq by expectation maximization (RSEM) were used to estimate the expression levels of all transcripts and to determine mRNA expression levels by calculating fragments per kilobase per million (FPKM). Differentially expressed genes (DEGs) were identified with $\log_2 | \text{fold-change} | > 1$ and FDR < 0.05 using the R package DESeq2 [38]. The GO database (http://geneontology.org) was used to reveal the biological functions of the DEGs. The KEGG database (http://www.genome.jp/kegg, accessed on 7 October 2021) was used to analyze the signaling pathways mainly involved in the DEGs based on the hypergeometric model. GO enrichment analysis of the DEGs was conducted using GOseq software. KOBAS software was used to identify the statistical enrichment of DEGs in the KEGG pathway analysis.

### 2.6. Real-Time Quantitative RT-PCR Validation

Ten DEGs with immune defense against Cu toxicity were chosen to validate the RNA-seq results. Their primers were designed on the sequences assembled by the transcriptome. These genes were analyzed using qRT-PCR, and the same RNA samples were used for transcriptome analysis. PCR was conducted using the SYBR Green Real-time PCR Kit according to the manufacturer's instructions. Previous studies showed that the *β-actin* gene is a suitable housekeeping gene for *A. woodiana* [39–41]. We also verified that the *β-actin* gene expression is stable in *A. woodiana*. We judged that using the β-actin as an internal control or using the mean of *β-actin* and elongation factor 1-alpha (another housekeeping gene commonly used in freshwater mussels [42]) as an internal control should not significantly affect the trend of the DEGs expression. Hence, the *β-actin* gene was used as an internal control to normalize the expression levels of the target genes. The PCR was carried out in a Roche LightCycler® 480 type II system. The thermal profile for qPCR was 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 1 min. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method [37,38]. Each qRT-PCR primer sequence is shown in Table 1.

**Table 1.** Details of the primer sequence used for qRT-PCR.

| Gene Name | Primer Sequence | Amplification Efficiency (%) |
|---|---|---|
| *Cullin-3* | F-AGGGCGACACACTATTTGGA<br>R-CCTGCTCCCTCAAGTAACCA | 98 |
| *RING finger protein B* | F-TGGAAAGCACACAAGTTGCA<br>R-GCTTGCCAGTTTCTTCTCGT | 97 |
| *Caspase-1* | F-GCATGCCAGGGACAAAAGTT<br>R-AGAGCTTGAACGAACCAGGA | 94 |
| *Inhibitor of apoptosis 1* | F-TCCACATGAGGTTCACACGA<br>R-CGCACAAAGGTTCGAATTGC | 100 |
| *Caspase* | F-CACAATACCTCTGAAGCCGC<br>R-AGGACTGGTTGTGATGAGCA | 102 |
| *Ubiquitin-conjugating enzyme E2-24 kDa* | F-CTTTGGTCCAGCACTGCAAT<br>R-CGTGGGAAGAAAAGAGCTCG | 97 |
| *E3 ubiquitin-protein ligase arc-1* | F-CTGACATGCCCAGTTTGCTT<br>R-GATGTGCGCCTCATTCTCTG | 102 |
| *Apoptosis regulator CED-9* | F-TCAGTGTATGGTATCGGGCC<br>R-CACTTTCACGCCCTCTGAAC | 105 |
| *Death-associated inhibitor of apoptosis 2* | F-TCGGGATGCCTGTATTGTGT<br>R-AGCTCTTTACTCCCTACCGC | 100 |

**Table 1.** *Cont.*

| Gene Name | Primer Sequence | Amplification Efficiency (%) |
|---|---|---|
| *Probable serine/threonine protein kinase drkD* | F-TCACTGCTGCCCGAATCATA<br>R-AGTGACACGGAAGACAGGAG | 103 |
| *β-actin* | F-GTGGCTACTCCTTCACAACC<br>R-GAAGCTAGGCTGGAACAAGG | 101 |

### 2.7. Statistical Analysis

Concentrations of Cu and other major ions were expressed as mean ± SD. Differences in Cu and major ion concentrations between the control and Cu-treated groups were analyzed using the independent samples *t*-test in SPSS 22.0 (IBM Corp., New York, NY, USA). Pearson's correlation was used to investigate the relationship between gene expression identified by qRT-PCR and RNA-seq. Statistical significance was set at $p < 0.05$. Additionally, the bioaccumulation efficiency of Cu in *A. woodiana* gills was evaluated using two parameters. First, the bioaccumulation capacity (BAC) was the ratio of the Cu concentration in the gills of the Cu-treated group to the concentration detected in the gills of the control group [43]. Second, the bioconcentration factor (BCF) was the ratio of the Cu concentration in the gills of the Cu-treated group to the concentration in water [44].

## 3. Results

### 3.1. Cu Bioaccumulation

The 'background' concentrations of Cu in *A. woodiana* gills were 1.3 ± 0.5 μg/g d.w. Copper concentrations in *A. woodiana* gills of the control and Cu-treated groups were 0.7 ± 1.2 μg/g d.w. and 331.9 ± 19.8 μg/g d.w., respectively. Cu concentrations in the Cu-treated group were significantly higher than those in the control group ($t = -28.961$, $p < 0.05$). Based on BAC, the mussels in the Cu-treated group were found to have bioaccumulated Cu in their gills to a level that, on average, was 474 times higher than that detected in the control group. The BCF values reached 161.9 ± 9.6.

### 3.2. Ion Concentrations

Ion concentrations in *A. woodiana* gills of both the control and Cu-treated groups generally ranked in decreasing order of $Ca^{2+} > Mg^{2+} > Na^{+} > K^{+}$ (Figure 1). However, the $Na^{+}$ ($t = 6.346$, $p < 0.05$) and $Mg^{2+}$ ($t = 3.135$, $p < 0.05$) concentrations decreased significantly after Cu exposure, with mean losses of 82% and 17%, respectively (Figure 1). There were no significant differences in $K^{+}$ and $Ca^{2+}$ concentrations between the two groups (Figure 1).

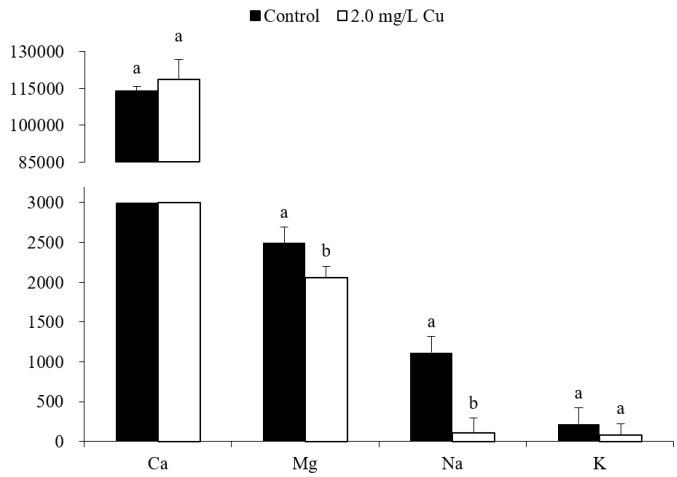


**Figure 1.** Ion concentrations in the gills of *Anodonta woodiana* from the control and Cu-treated groups. Data are presented as mean ± SD of three parallel measurements. Values with different letters indicate a significant difference (*t*-test, $p < 0.05$) between the control and Cu-treated groups.

### 3.3. Histopathology

Gill sections of *A. woodiana* stained with hematoxylin and eosin from the control and Cu-treated groups are shown in Figure 2. The transverse and longitudinal sections show that the gills in the control group exhibited well-preserved structures, and gill filaments were covered with ciliated epithelium on their external surfaces (Figure 2A,B). In contrast, gills from the Cu-treated group exhibited clear histological alterations. The gill filaments lost most of their cilia, desquamation started in the epithelium layer, and atrophy of the gill filaments was observed (Figure 2C,D).

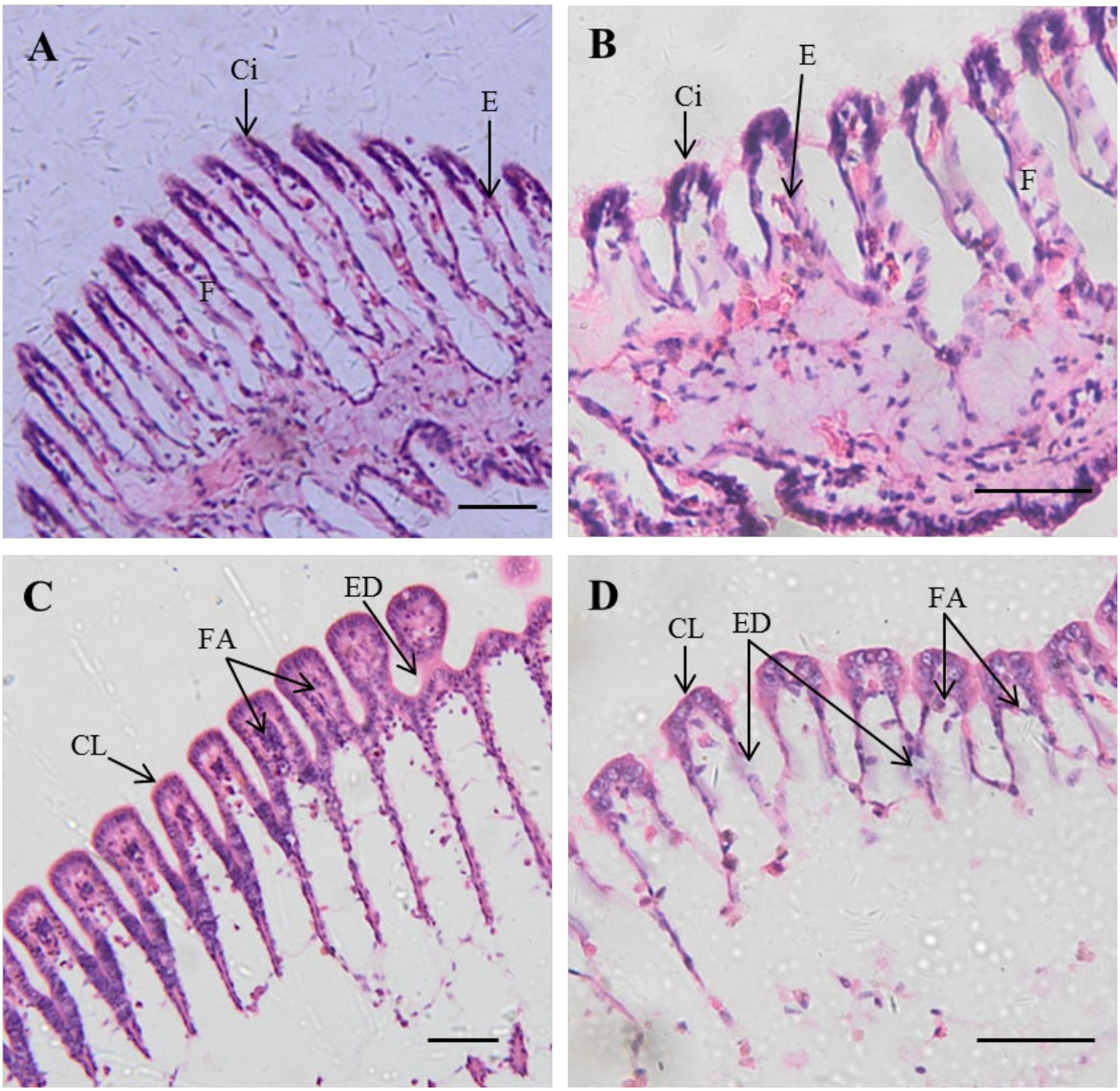


**Figure 2.** Light micrographs of transverse and longitudinal gill sections of *Anodonta woodiana* revealed the histological structure of the control (**A**,**B**) and Cu-treated (**C**,**D**) groups. Ci: cilia, E: epithelium, F: filament, CL: cilia loss, ED: epithelium desquamation, FA: filament atrophy. Scale bar: 50 μm.

### 3.4. Differential Expression Analysis

A total of 3160 genes were differentially expressed in the gills of the Cu-treated group compared to in those of the control group, including 1870 upregulated and 1290 downreg-

ulated genes (Figure 3). To confirm the veracity and reliability of the DEGs identified by RNA-seq, 10 genes were selected for qRT-PCR analysis. As shown in Figure 4, the qRT-PCR expression patterns of these genes were in agreement with the results of the RNA-seq analysis ($R^2 = 0.958$, $p < 0.05$).

To obtain insight into the biological functions that can be differentially regulated by Cu exposure, GO enrichment analysis was performed, comparing the Cu and control groups. The GO enrichment analysis included three major functional categories: biological processes, cellular components, and molecular functions. Cellular processes, metabolic processes, biological regulation, and responses to stimuli contained the most DEGs in biological processes (Figure 5). The cell, cell part, organelle, and membrane contained the most DEGs among the cellular components (Figure 5). Binding and catalytic activity contained the most DEGs in terms of molecular functions (Figure 5).

The KEGG database was used to compare the differentially expressed UniGene sequences. In comparison, the Cu-treated group induced eight significantly enriched pathways, including apoptosis, arginine and proline metabolism, the toll-like receptor signaling pathway, apoptosis-multiple species, histidine metabolism, beta-alanine metabolism, cytokine–cytokine receptor interaction, and the p53 signaling pathway (Figure 6).

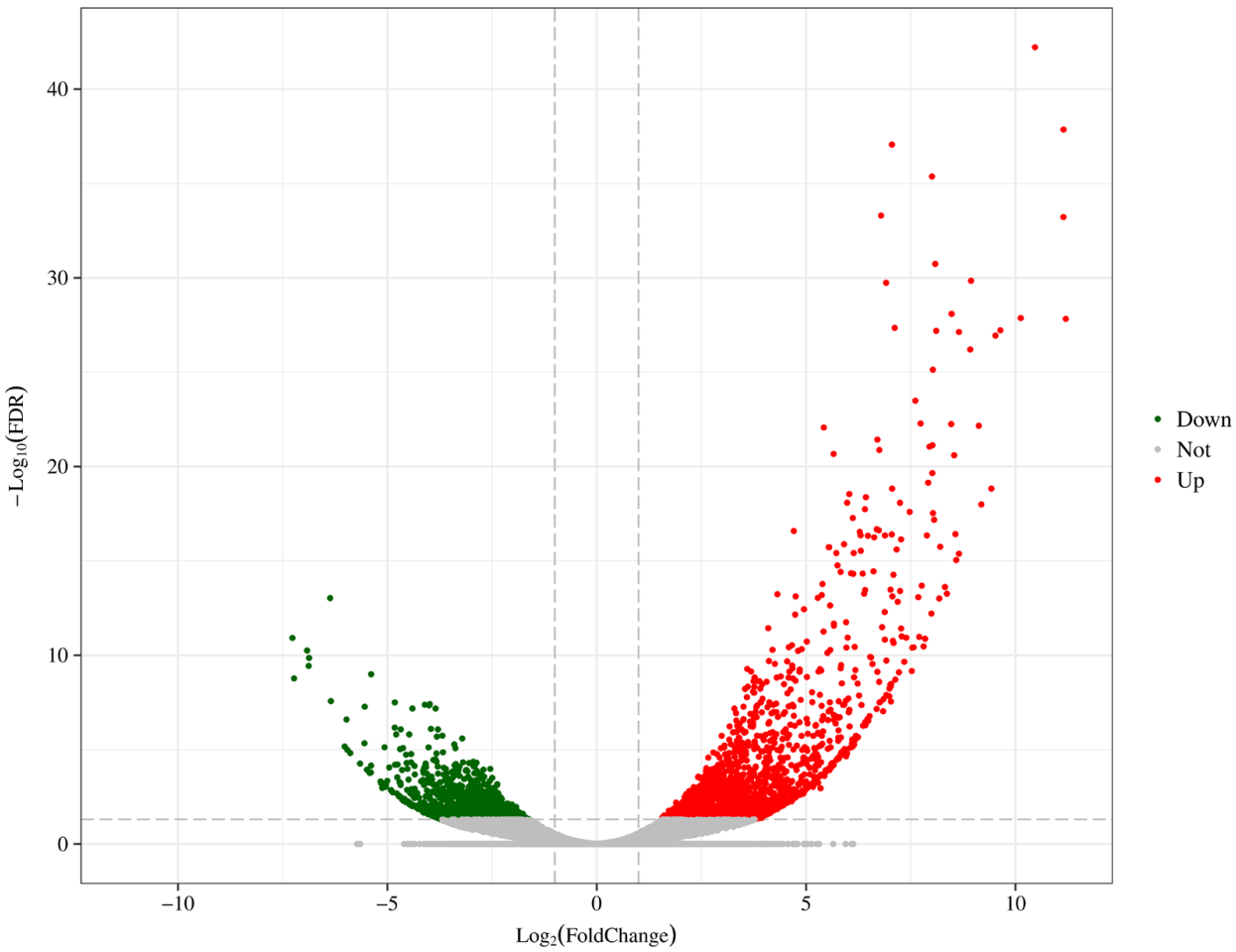


**Figure 3.** A volcano plot of differentially expressed genes in the Cu-treated group compared to the control group. Red and green dots represent upregulated and downregulated genes, respectively.

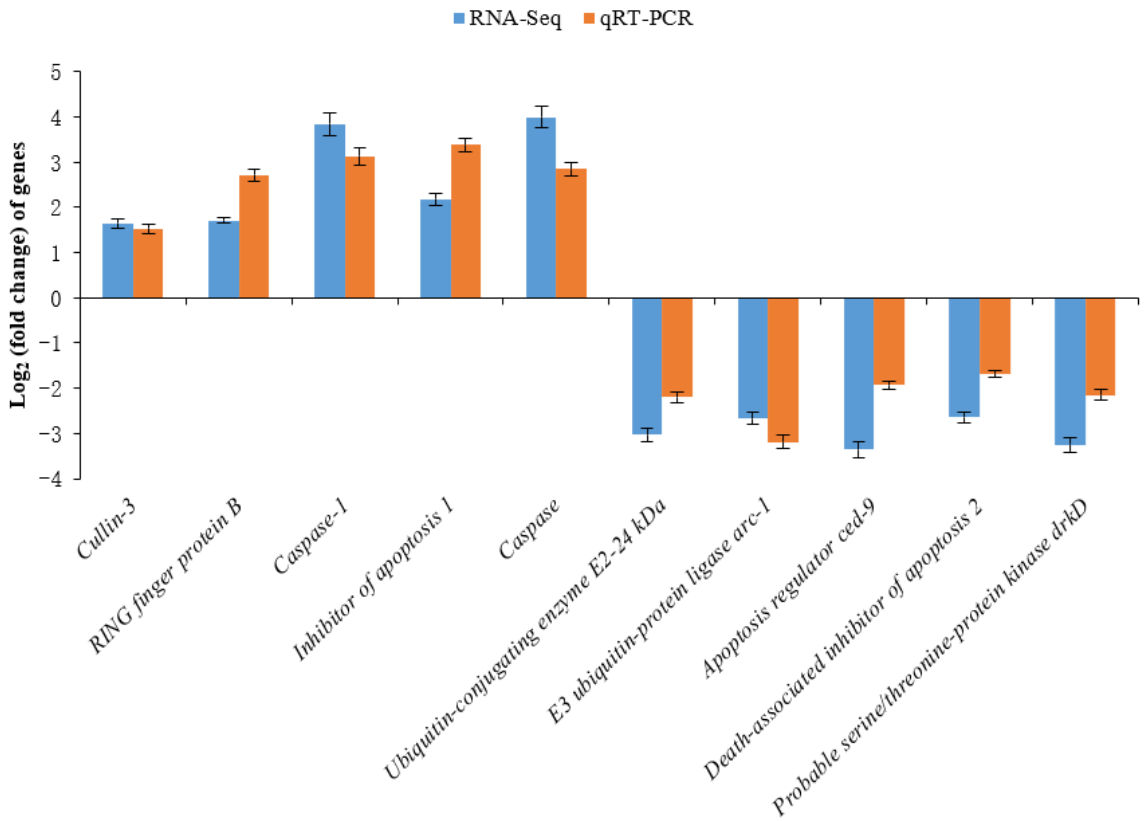


**Figure 4.** Comparison of gene expression patterns obtained using RNA-seq and qRT-PCR. The Trinity-assigned genes were annotated using the NR database to obtain the gene names. The X-axis displays 10 selected genes, and the Y-axis represents the relative fold change.

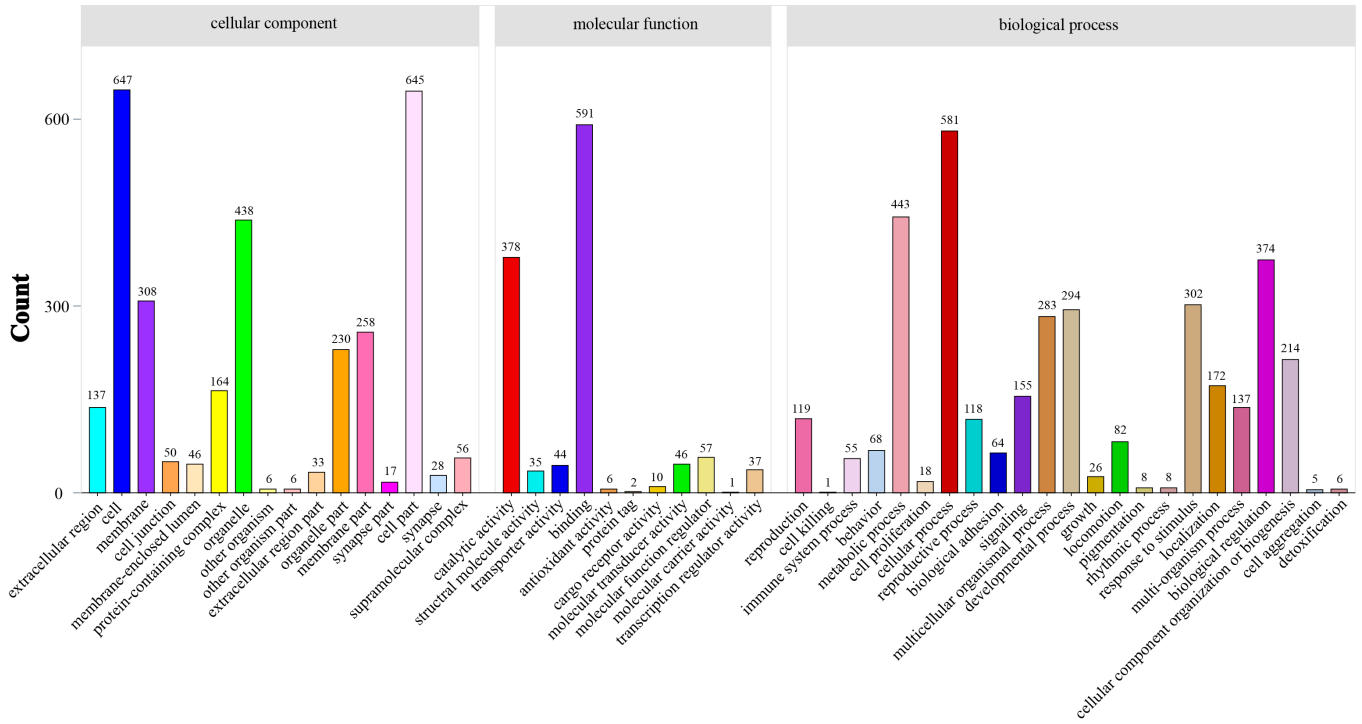


**Figure 5.** Gene ontology (GO) classifications of differently expressed genes.

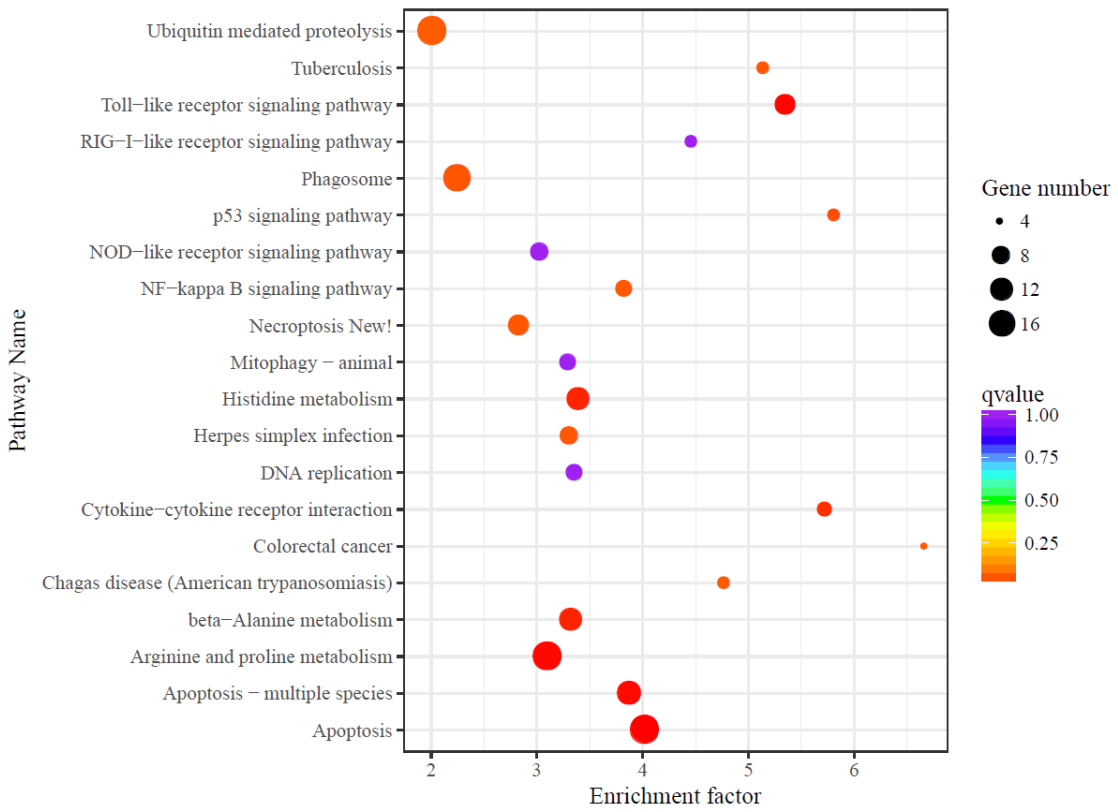


**Figure 6.** Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment of differentially expressed genes (DEGs) after 2.0 mg/L Cu exposure for 72 h. The vertical axis represents different pathways, and the horizontal axis represents the richness factor. Color shades correspond to different q-values, and dot size represents the number of DEGs.

## 4. Discussion

### *4.1. Cu Bioaccumulation*

Gills play an important role in respiration and in feeding in filter-feeding species. In *A. woodiana*, the filtration rate was about 260 L/h/kg d.w. of soft tissue [34]. Aqueous Cu may enter the mussel gills during filtration via facilitated diffusion, active transport, or endocytosis [45]. Therefore, mussel gills are used as indicator tissues to study the bioaccumulation of aqueous Cu. For example, in duck mussel *Anodonta anatina* exposed to 0.3 µmol/L aqueous Cu for 24 d, approximately 70% of the Cu accumulated in gills with a peak concentration of 75 µmol Cu/kg (4.7 µg/g d.w.); the BAC was 6.5 [46]. In the present study, Cu concentrations in the gills of *A. woodiana* of the Cu-treated group reached 331.9 ± 19.8 µg/g d.w., and the BAC reached 474. Both Cu concentrations and the BAC of *A. woodiana* gills in the present study were higher than those of *A. anatina* [46]. Moreover, the BCF of *A. woodiana* gills exposed to Cu at 72 h was up to 161.9. These results suggest that *A. woodiana* gills might have a high bioaccumulation capacity for aqueous Cu.

### *4.2. Ionoregulatory Disturbance*

$Na^+$, $Mg^{2+}$, $K^+$, and $Ca^{2+}$ are essential for freshwater mussels, with varied functions, for example, as electrolytes, enzyme constituents, and building materials [47]. A deficiency in these ions leads to serious disorders, such as inhibition of $Na^+/K^+$-ATPase activity, thereby reducing osmolarity regulation. In the present study, we found that aqueous Cu exposure/bioaccumulation significantly decreased $Na^+$ and $Mg^{2+}$ concentrations in the gills of *A. woodiana*. As documented in freshwater mussel *L. cardium* larvae, $Na^+$ and $Mg^{2+}$ concentrations reduced by 30% and 40% after 0.11 µmol/L or 0.27 µmol/L Cu acute exposure for 48 h, paralleled by 20% and 50% mortality, respectively [48]. Freshwater

mussel *L. siliquoidea* juveniles were chronically exposed (28 d) to 2 μg/L or 12 μg/L Cu and mainly exhibited $Na^+$ concentration losses of up to 50% and 70%, respectively; this resulted in 21% and 70% mortality, respectively [49]. Aqueous Cu competes with the $Na^+/H^+$ exchanger isoform (NHE-2) for $Na^+$ binding sites [50] and inhibits the activity of $Na^+/K^+$-ATPase [51], leading to a significant decrease in $Na^+$ concentrations in freshwater organisms [50,51]. In addition, aqueous Cu exposure inhibits $Mg^{2+}$-ATPase activity in freshwater mussels [52], which might explain the decreased $Mg^{2+}$ concentration in the gills of *A. woodiana*. Considered together, we conclude that increased bioaccumulation of Cu can induce ionoregulatory disturbances in freshwater mussels and further threaten their survival.

### 4.3. Histological Hazards

Histopathological observation is an effective method for studying Cu toxicology in mussels [43,53]. For example, the freshwater mussel *Physa acuta* bioaccumulates elevated levels of Cu in the gills but lower amounts in the digestive tract, muscles, and digestive glands [43]. Accordingly, the most serious histopathological changes are observed in the gills, where mucus cells increase, cilia are lost, and columnar cells degenerate in the epithelium [43]. In this study, high bioaccumulation of Cu seemed to induce more serious histological damage than that observed in *P. acuta*, involving cilia loss, epithelial desquamation, and filament atrophy [43]. From the histology, we propose that triggering cell death by disrupting cell structure and function may be a Cu-specific toxic pathway. Previous studies have also shown that Cu toxicity can cause cell death by damaging the cytoskeleton [54] and disrupting mitochondrial function [55]. Histological damage can affect vital physiological functions such as respiration, filtration, nutrient uptake, and ionic regulation of the gills, which can subsequently affect the growth and survival of freshwater mussels [56]. In freshwater ecosystems, serious Cu contamination events occur frequently [4–6], so it is important to pay more attention to the impact of Cu contamination on freshwater mussel resources.

### 4.4. Transcriptome Responses

Molecular changes can sensitively reflect Cu toxicity [57]. For instance, 3549 DEGs were detected in the gills of *Mizuhopecten yessoensis* exposed to 0.1 mg/L Cu for 72 h, with 1312 upregulated and 2237 downregulated genes [30]. In contrast, most DEGs were upregulated in *A. woodiana* gills after aqueous Cu exposure. This implies that gene expressions of *A. woodiana* were largely activated by aqueous Cu exposure and that the molecular mechanisms of mussels responding to Cu toxicity might be species specific. In addition, these DEGs have the potential to be used for the development of biomarkers of Cu contamination in freshwater ecosystems using *A. woodiana* as a bioindicator.

GO enrichment analysis showed that DEGs in *A. woodiana* gills exposed to Cu were mainly involved in cellular and metabolic processes, biological regulation, and responses to stimuli. These generally differed from those observed in other mussels, such as *M. yessoensis* [30]. This indicates that, although mussels can metabolically fight Cu stress, such as by producing more antioxidants to scavenge intracellular reactive oxygen species (ROS) [27,30], excessive Cu bioaccumulation may cause damage to cell structure and biological function, even extending to histological damage. The molecular toxicity of Cu was confirmed by the gill histological abnormalities, including cilia loss, epithelial desquamation, and filament atrophy, triggered by Cu exposure in this study.

KEGG pathway enrichment analysis showed that DEGs were significantly enriched in pathways related to oxidative stress (the toll-like receptor signaling pathway and cytokine–cytokine receptor interaction), apoptosis (apoptosis, apoptosis-multiple species, and the p53 signaling pathway), and metabolism (arginine and proline metabolism, histidine metabolism, and beta-alanine metabolism). The toll-like receptor signaling pathway is an evolutionarily conserved innate immune pathway that plays a key role in detecting non-self-antigens and immune system activation [30]. Cytokine–cytokine receptor interactions

are related to immune defenses [58]. Both the toll-like receptor signaling pathway and cytokine–cytokine receptor interaction may be induced by excessive ROS [59]. These results indicate that Cu bioaccumulation may stimulate ROS generation and activate the mussel's immune defense. Apoptosis is an important immune response in mussels [60]. It enables the adequate clearance of damaged, senescent, and infected cells without inflammation [60]. Environmental stimulation can induce apoptosis in the cells of mussels [60,61]. For instance, a sudden drop in salinity from 30‰ to 22‰ significantly increased apoptosis and apoptosis-multiple species signaling pathways in the ark shell *Anadara kagoshimensis* [61]. The p53 signaling pathway is significantly enriched in aquatic organisms, indicating that DNA damage might occur [62]. If DNA damage is extensive, p53 triggers apoptosis [63]. Here, we suggest that Cu toxicity can induce DNA damage and further trigger apoptosis in mussel gills. Amino acids such as histidine and beta-alanine play a substantial role in energy metabolism [64]. Additionally, changes in arginine and proline metabolism are important signatures of apoptosis [65]. The metabolism of eight amino acids (phenylalanine, methionine, histidine, glutamic acid, tryptophan, cysteine, glycine, and alanine) in the hemolymph of the green-shell mussel *Perna canaliculus* was previously shown to be significantly affected by Cu stress [65]. This indicates that aqueous Cu bioaccumulation may also affect the amino acid metabolism of *A. woodiana* and may further affect the survival of freshwater mussels.

## 5. Conclusions

To the best of our knowledge, this study was the first comprehensive screening of the effects of aqueous Cu bioaccumulation on ionoregulatory homeostasis, histological features, and transcriptome responses using *A. woodiana* gills as the indicator tissue. High bioaccumulation of Cu induced ionoregulatory disturbances ($Na^+$ and $Mg^{2+}$ losses) and remarkable histological alterations (cilia loss, epithelium desquamation, and filament atrophy). Additionally, the molecular mechanisms of mussel responses to Cu toxicity might be species specific. In *A. woodiana*, most DEGs were activated by Cu bioaccumulation. These DEGs were mainly involved in cellular processes, metabolic processes, biological regulation, and response to stimuli and were significantly enriched in oxidative-stress-, apoptosis-, and metabolism-related pathways. These results provide valuable insights into Cu toxicity mechanisms in freshwater mussels and benefit the conservation of freshwater mussel resources. In future studies, the DEGs and their enrichment pathways from transcriptome analysis could be used as candidate biomarkers for monitoring Cu contamination in freshwater ecosystems.

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