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Physiological and Transcriptomic Response of Asiatic Hard Clam *Meretrix meretrix* to the Harmful Alga *Heterosigma akashiwo*

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Abstract: The ichthyotoxic raphidophyte *Heterosigma akashiwo* is associated with harmful algal blooms (HABs). Although the influence of *H. akashiwo* on fish has been reported, little is known of its effects on other marine organisms, such as shellfish. In this study, the physiological and transcriptomic responses of the Asiatic hard clam *Meretrix meretrix* to *H. akashiwo* were investigated. The results showed that clam survival was not influenced by *H. akashiwo* (at a concentration of 2×10^4 cells/mL), and *M. meretrix* eliminated the alga effectively after 48 h in co-culture. *H. akashiwo* did not alter the antioxidant capacity of the clams. However, 544 unigenes were found to be differentially expressed between the control and *H. akashiwo*-treated clams. Interestingly, the significant up-regulation of lysosome-related genes and transport proteins suggested remodeling of the clam metabolism possibly for digestion of *H. akashiwo*. In summary, our results indicate that the clam *M. meretrix* can effectively feed on *H. akashiwo*, and intensification of shellfish farming may be helpful to control and mitigate *H. akashiwo* blooms.

Keywords: *Heterosigma akashiwo*; *Meretrix meretrix*; transcriptome; differentially expressed genes

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1. Introduction

Microscopic algae in marine and fresh water not only form the foundation of the food web in providing nutrients for various aquatic organisms, such as filter-feeding shellfish and finfish larvae, but also channel carbon dioxide from the atmosphere to deeper water levels [1]. However, the proliferation and accumulation of certain algal species can cause serious damage to the ecosystem, as well as have repercussions on the economy and human health [2,3]. Such overgrowths are termed harmful algal blooms (HABs), frequently described as "red tides" due to color changes in the water caused by the proliferation of species such as dinoflagellates. It is estimated that HABs have caused economic losses of approximately 0.87 billion US dollars over the past three decades in China, resulting from the mass mortality of fish and shellfish and the negative impacts on tourism [4]. HABs can usually be divided into three main types according to their specific harmful attributes [5,6]. The first type of HAB produces toxins that are harmful to invertebrates, fish, shellfish, and other aquatic species. Although these toxins may not necessarily kill the predator directly, or may even show minimal effects on the predator, they can be transferred and concentrated through the food web, causing illnesses or death when human consumers eat contaminated fish or shellfish. The second type of HAB is non-toxic but causes damage to environmental ecosystems through excessive biomass accumulation, which can shade other phytoplankton and sea grass, reducing photosynthesis, and also increase bacterial

respiration during the decay of the bloom, leading to overall oxygen depletion and severe environmental consequences. The third type of HAB is also non-toxic to humans but harms invertebrates and fish by clogging fish gills or stimulating the production of specific compounds (e.g., reactive oxygen species and hemolytic substances), with negative impacts seen particularly in intensive aquaculture systems.

Based on the available literature, the raphidophyceae *Heterosigma akashiwo* (Hada) Hada ex Y. Hara et Chihara should be classified in the third HAB category. It is responsible for massive red tides and is distributed worldwide [7–9]. This species is notorious because *H. akashiwo* blooms cause mortality in caged fish although the mechanisms underlying this toxicity remain controversial [8,10–12]. Although the effects of *H. akashiwo* have been investigated in fish, little is known of its effects on other marine organisms. In marine environments, the first organisms affected by HABs are filter-feeding shellfish (e.g., bivalves), which form the bulk of the primary consumers of microalgae [13]. A previous report by Keppler et al. (2005) observed that short-term exposure of the southeastern oyster (*Crassostrea virginica*) to high concentrations of *H. akashiwo* had long-term adverse effects, including damage to the hepatopancreas [14]. Another study showed that while several harmful raphidophytes, including *H. akashiwo*, did not affect either the fertilized eggs or embryos of the Japanese pearl oyster (*Pinctada fucata martensii*), the effects on post-embryonic oysters were lethal [15]. Both cellular biomarkers and measurements of oxidative stress have been used to assess the harmful effects of *H. akashiwo* on shellfish [14–16]. However, the assessment of a few physiological parameter, such as antioxidant enzyme activity and lysosomal destabilization, does not fully reflect the extent of the shellfish response to HAB species exposure [14,17]. This matter can now be resolved to some extent using transcriptome profiling. For instance, Mat et al. (2018) found that the transcriptome response of the Pacific oyster *Crassostrea gigas* to dinoflagellate *Alexandrium minutum* exposure was related to toxins inducing paralysis in the shellfish [18].

The clams *Meretrix meretrix* is an important commercial bivalve in South and Southeast Asia coastal waters [19]. Previous studies showed that several HAB species have toxic effects on *M. meretrix* [4,20]. For instance, Xu et al. (2017) found that bloom-forming dinoflagellate *Akashiwo sanguinea* caused extensive mortalities in multiple aquatic animals, including *M. meretrix* [20]. Nevertheless, little is known about ichthyotoxic *H. akashiwo* effects on *M. meretrix*. In this study, the juvenile *M. meretrix* was exposed to *H. akashiwo* for 48 h. Various physiological parameters, including superoxidase dismutase (SOD) activity, total antioxidant capacity (T-AOC), and the malondialdehyde (MDA) content, were examined. In addition, the transcriptome response of *M. meretrix* after exposure to *H. akashiwo* was determined by Illumina sequencing. The relationships between the clam *M. meretrix* and *H. akashiwo* are also discussed.

2. Materials and Methods

2.1. Heterosigma Akashiwo Culture

Heterosigma akashiwo cultures (strain: CCMA369) were obtained from the Center for Collection of Marine Algae at Xiamen University. The strain was originally isolated from the East China Sea on 20 May 2011. The algae were grown in f/2-Si medium prepared with natural seawater and filtered through 0.22-µm membranes before autoclaving [21]. Cultures were maintained at 20 °C, with an average photon flux of 100 µE m^{-2} s^{-1} under a 14: 10 h light: dark cycle.

2.2. Meretrix Meretrix Maintenance

The juvenile *Meretrix meretrix* were obtained from a shellfish farm in Dongying, China. Then, individuals without shell damage and vigorous (extension of siphons and foot) were selected, which with shell lengths of 8.5–11.6 mm In the laboratory, the clams were kept under regular culture conditions at 20–21 °C in natural seawater. The clams were fed powdered *Spirulina platensis* microalgae. Briefly, algae power was dispersed in distilled water, and algal suspension was added to into experimental tanks two times per day with

a final concentration of about 3×10^4 cells/mL. Clams were acclimatized to the laboratory environment for two weeks.

2.3. Exposure Experiments and Sample Collection

After two weeks of acclimation, the clams were randomly divided into two groups, one of which was exposed to 2×10^4 cells/mL of *H. akashiwo*. Clams without any treatment were set as control. Each group was stocked in triplicate one-liter containers, each containing 50 clams. In addition, three containers had *H. akashiwo* (2×10^4 cells/mL) only. At 48 h, the concentration of *H. akashiwo* in the containers (Treatment) decreased by over one order of magnitude. This indicate that the *H. akashiwo* were being consumed by clams. Thus, sampling was conducted after 48 h exposure. Briefly, thirty clams were randomly selected from each group and euthanized by freezing. Digestive gland tissue from these clams were mixed together to make a sample. Part of the sample was frozen at −40 °C for further analysis of antioxidant enzymes and the remaining sample was immediately snap-frozen in liquid nitrogen and stored at −80 °C for future RNA extraction. *H. akashiwo* cell densities were assessed by counting in a Sedgwick-Rafter chamber under a microscope.

2.4. Measurements of the Antioxidant Enzyme Activities

Samples were homogenized in sterile PBS (pH 7.4). The total protein concentrations were measured using a BCA (bicinchoninic acid) kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The total antioxidant capacity (T-AOC) was determined by colorimetric assays and expressed as the millimolar Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) equivalent calculated using a standard curve as specified by the kit instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). In addition, superoxidase dismutase (SOD) activities and malondialdehyde (MDA) contents were measured using their respective assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the provided directions.

2.5. RNA Sequencing, De Novo Transcriptome Assembly, and Functional Annotation

Total RNA was extracted using TRIzol. The integrity of RNA was initially evaluated by agarose gel electrophoresis. Furthermore, RNA quality and quantity were assessed using the Agilent RNA 6000 Nano Kit of the Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA, USA). One microgram of total RNA from each sample was used for library construction. Briefly, mRNA was extracted from the total RNA with polyT oligo-conjugated magnetic beads and was fragmented using fragmentation buffer. A TruSeq RNA kit (Illumina, San Diego, CA, USA) was used for high-throughput sequencing library preparation, according to the provided directions. Sequencing was performed on an Illumina Novaseq platform by Novogene Co., Ltd. (http://www.novogene.com/) (accessed on 15 December 2020), followed by synthesis of the paired-end reads.

2.6. Transcriptome Assembly and Annotation of Differentially Expressed Genes (DEGs)

Reads containing poly-N and adapters, as well as low-quality reads were filtered out to obtain the clean reads. The transcriptome was assembled using Trinity with a min_kmer_cov value of 2 and default values for other parameters, a reference transcriptome was generated from six samples [22]. Genes were identified by BLAST against the non-redundant NCBI and Swiss-Prot databases. Annotation and pathway enrichments of the genes were evaluated using GO (Gene Ontology), KEGG (Kyoto Encyclopedia of Genes and Genomes), and KO (KEGG Orthology) [23,24]. GO and KEGG enrichment analysis were conducted using the clusterProfiler R package. Manual annotation of the most relevant genes was performed in BLAST.

Bowtie 2 was used for read mapping to the reference transcriptome, and quantification was conducted with RSEM v1.2.15 [25]. Differential expression of genes between the control and *H. akashiwo*-exposed groups was performed with the DESeq2 package in R (1.20.0) [26]. The Benjamini and Hochberg approach was used for *p*-value adjustment. Only the genes

with *p*-values (*padj*) < 0.05 and Log_2 |fold change| > 1 were considered to be differentially expressed and were used for further analyses.

2.7. Identification of Core Genes and Verification of DEGs

Core genes were defined as those showing both high and stable expression [27]. Here, genes with expression ≥ 90th percentile FPKM (Fragments Per Kilobase of mRNA sequence per Million mapped reads) and coefficients of variation (CV) ≤ 0.08 across six samples (three control, three treatment) were collected. After that, Log_2 (FPKM) was used for calculating the relative expression levels of genes, and the average expression stability values (M) were determined using the geNorm algorithm [28]. For further selection of the most stable core genes, ten genes with the lowest M values were ranked, and the gene numbers required for normalizing target gene expression levels were also determined.

For further verification of the DEGs in the transcriptome, DEG read counts were normalized to those of the stably expressed genes described above. The stable gene normalized counts (SGNCs) were determined as previously described (Table S3) [9,29]. Here, the concept of SGNC calculation is similar to that used in normalization against reference genes in quantitative real-time PCR (qRT-PCR).

3. Results

3.1. Exposure of Clams to H. akashiwo

As shown in Figure 1a, the 48 h survival rates for clams in the control and *H. akashiwo*-treated groups were 96.7% and 97.3%, respectively. This indicated that *H. akashiwo* short-term exposure did not significantly impact *M. meretrix* survival. Consistent with this, clams exposed to *H. akashiwo* also appeared to open their shell valves fully. In addition, the cell densities of *H. akashiwo* were monitored after 48 h of incubation in the experimental containers, both with and without clams. In the without clams groups, there was little change in the concentrations of *H. akashiwo*. In contrast, there was a significant decrease of *H. akashiwo* concentrations after 48 h in the treatment groups where clams were added (Figure 1b). These results indicated that the *H. akashiwo* cells were efficiently filtered by *M. meretrix* while the survival of the clams remained unaffected.

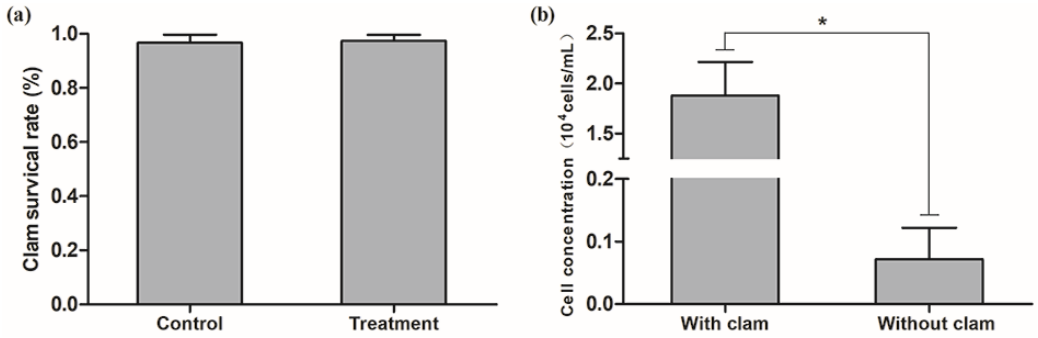

Figure 1. Effect of *Heterosigma akashiwo* exposure on the survival rate of the clam *Meretrix meretrix* (**a**). Changes in *Heterosigma akashiwo* concentrations under different conditions (**b**). Error bars represent standard deviations (n = 3). Asterisk (*) represents that cell concentration of *Heterosigma akashiwo* was significantly different between two groups ($p < 0.05$).

3.2. Effect of H. akashiwo on Clam Antioxidant Functions

In this study, three physiological and biochemical parameters were measured to determine the effect of *H. akashiwo* on clams. Compared with the control groups, there were no significant changes in the T-AOC levels and SOD activities in the clams (Figure 2a,b). Although the MDA levels were slightly lower in the treatment group compared to those in the control, this difference was not significant (Figure 2c). The results showed that *H. akashiwo* 48 h exposure did not influence the antioxidant capacity of the clams.

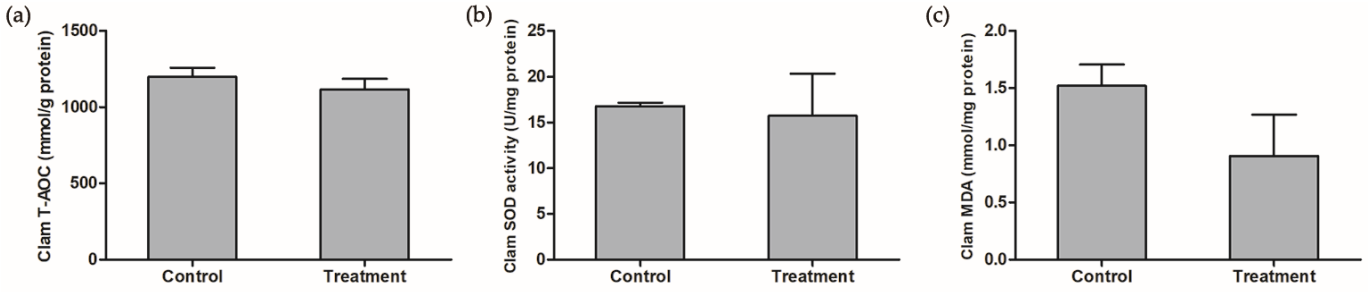

Figure 2. Antioxidant response of the clam *Meretrix meretrix* after exposure to *Heterosigma akashiwo* for 48 h. (**a**) Total antioxidant capacity (T-AOC); (**b**) superoxidase dismutase (SOD) activity; (**c**) malondialdehyde (MDA) content. Error bars represent standard deviations (n = 3).

3.3. Transcriptome Profiling and DEG Identification

The RNA-seq yielded a total of 130,247,936 clean reads, with an average of 21,707,989 clean reads per sample (Table S1). All raw data of RNA-seq were submitted to NCBI SRA database (BioProject number: PRJNA860230). Assembly of the clean reads resulted in the identification of 112,317 unigenes with a mean length of 1035 bp. Of these, 44,953 transcripts (39.70%) were annotated in at least one database (e.g., Swiss-Prot and NCBI non-redundant protein sequences). Approximately 73.23% of the total clean reads mapped to the assembled reference transcriptome.

As shown in Figure 3, a total of 544 unigenes (Log_2 | fold change | > 1 and $p < 0.05$) were classified as DEGs. Of these, 283 were upregulated and 262 were downregulated. To further explore the response of *M. meretrix* after *H. akashiwo* exposure, the DEGs were analyzed in terms of KEGG pathway enrichment. This showed significant enrichment in 13 pathways ($p < 0.05$) of which the most significant was the "regulation of actin cytoskeleton" although the corrected *p*-value was > 0.05. Furthermore, the 283 upregulated DEGs were enriched in the ribosome, lysosome, AMPK signaling pathway, oxytocin signaling pathway, mineral absorption, and basal transcription factors ($p < 0.05$) (Figure S1a) while the downregulated DEGs were enriched in 12 pathways (e.g., regulation of actin cytoskeleton and focal adhesion) ($p < 0.05$) (Figure S1b). Specifically, we observed significant up-regulation of three unigenes, namely, NPC intracellular cholesterol transporter 2 (NPC2), ganglioside GM2 activator (GM2A), and clathrin light chain A (CLTA), involved in the lysosomal pathway (Figure 4). The variation in types of enriched pathways suggests that the metabolism of the clams was remodeled after *H. akashiwo* exposure.

3.4. Verification of DEGs

In this study, 90 genes were classified as core genes due to their elevated expression and stability under two different conditions. The expression of these genes ranged from 38.89 to 7087.29 FPKM, with an average of 327.72 FPKM. Fifty-seven of the genes were annotated in Swiss-Prot (Table S2). Furthermore, after calculation of the M values of the annotated core genes by geNorm, the 10 most stable genes were selected. As shown in Figure 5, sodium/potassium-transporting ATPase subunit beta (ATPA1) and mitochondrial-processing peptidase subunit beta (PMPCB) were found to be most stable, with M values lower than the threshold value of 1.5. The geNorm program was also used to determine the optimal numbers of housekeeping genes for normalization. It was then found out that the two core genes with the highest stability, namely, *ATPA1* and *PMPCB*, were sufficient for the normalization of *M. meretrix* gene expression.

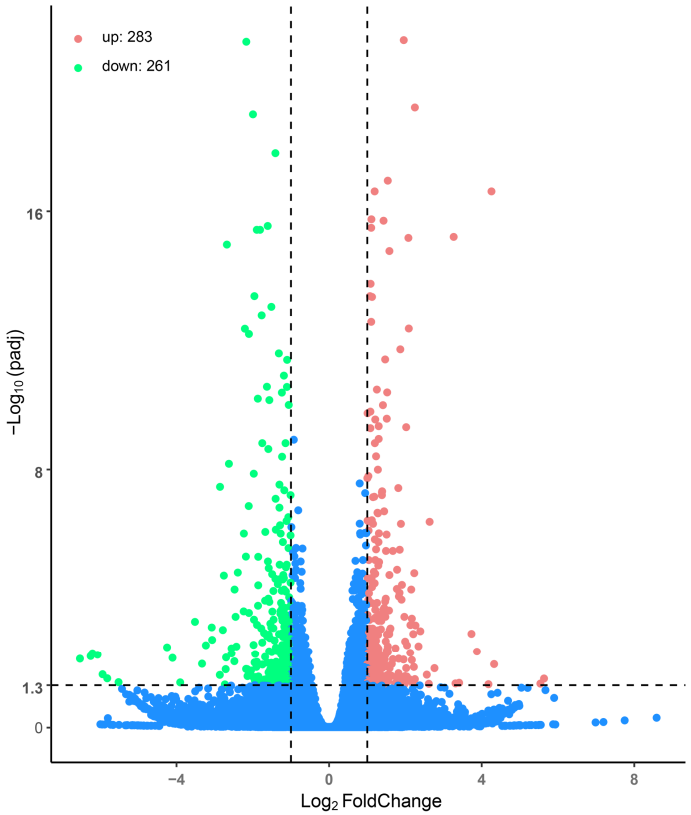

Figure 3. Volcano plot of differentially expressed genes (DEGs) in the clam *Meretrix meretrix* between the control and treatment groups. Red dots represent up-regulation after treatment; green dots represent down-regulation after treatment. DEGs were identified by Log_2 | fold change | > 1 and *p*-value (*padj*) < 0.05.

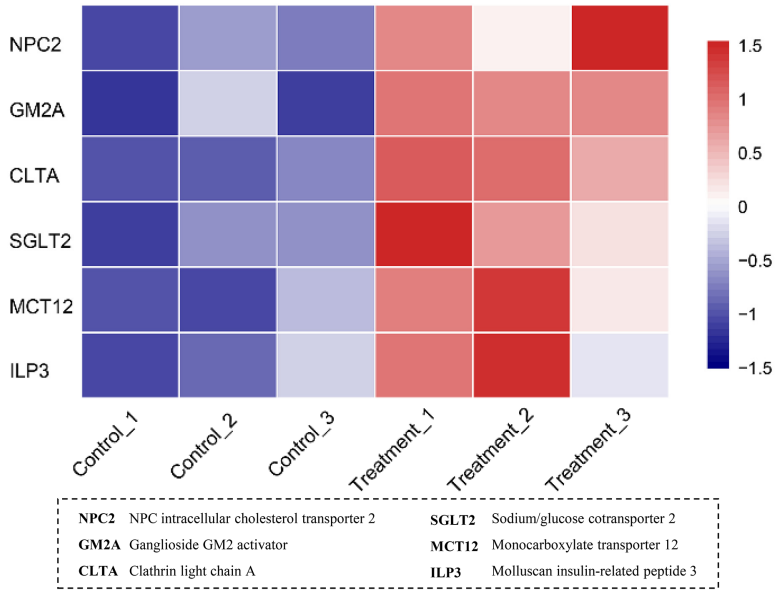

Figure 4. Heatmap showing the expression levels of the most significant differentially expressed genes after *Heterosigma akashiwo* exposure in the clam *Meretrix meretrix*. The color scale indicates the Row Z-score from the FPKM value.

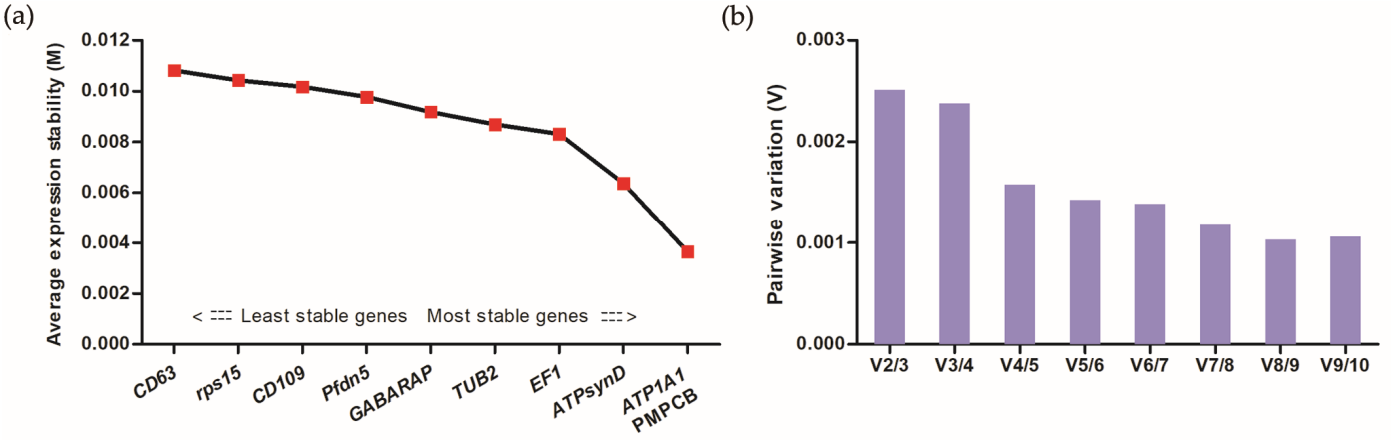

Figure 5. Stability of gene expression of ten core genes. (**a**) Ranking of the ten genes by geNorm. Left-hand side represents low stability; right hand-side represents high stability. (**b**) The optimal number of core genes for the normalization of gene expression was calculated by geNorm. Detailed information about these genes is given in Table S2.

To verify the differential gene expression detected by the transcriptome data, two stably expressed genes (*ATPA1* and *PMPCB*) were chosen to normalize the transcriptomic read counts. Six DEGs were selected for SGNC verification in *M. meretrix*. As shown in Figure 6, all these genes showed similar expression to that seen in transcriptome analysis. These results confirmed that the DEGs identified in the transcriptome were reliable. Nevertheless, identified DEGs still need to be verified using traditional qRT-PCR in the further investigation.

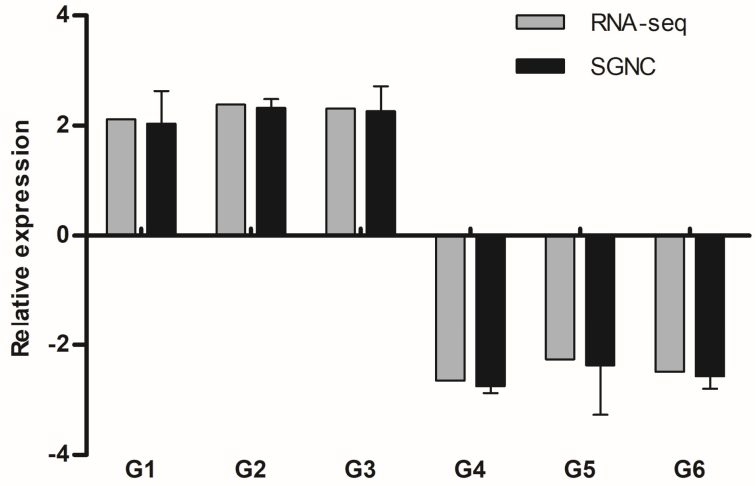

Figure 6. SGNC validation of differentially expressed genes. G1: molluscan insulin-related peptide 3; G2: clathrin light chain A; G3: monocarboxylate transporter 12; G4: tubulin beta-4 chain; G5: galectin-4; G6: cathepsin L. SGNC represents gene expression normalization to *ATPA1* and *PMPCB*. Relative expression values represent the fold change of gene expression in the treatment group compared with the control.

4. Discussion

Damage caused by *H. akashiwo* has been reported in many coastal waters [30,31]. However, the ichthyotoxic mechanisms of *H. akashiwo* is still unclear. To date, three hypotheses have been proposed to account for its effects on fish mortality: (1) the alga produces excessive amounts of mucus that adheres to the fish gill lamellae, leading to impairing both respiration and osmoregulation [32]; (2) the production of a neurotoxin (e.g., a brevetoxin-like toxin) directly causing the death of fish rather than physical in-

terference in gill function [33]; and (3) the generation of reactive oxygen species, such as superoxide, hydrogen peroxide, and hydroxyl radicals, by *H. akashiwo*, causing gill damage and resultant asphyxiation [8]. Here, the effects of *H. akashiwo* on the mollusk *M. meretrix* were investigated. The results showed that clam survival was not affected by *H. akashiwo* treatment. More importantly, the significant decrease in *H. akashiwo* cell concentrations suggested that the clams can easily feed off this alga. In addition, pigment deposition indicated digestion of *H. akashiwo* after ingestion (Figure S2). It has been reported that *H. akashiwo* can promote the growth of the Manila clam, *Ruditapes philippinarum* [34]. Interestingly, significant increases in the soft-tissue weight, body-mass index, and glycogen content of the clams were found when the clams were fed *H. akashiwo* compared to control (fed only *Chaetoceros neogracile*) [34]. Consistent with these reports, Basti et al. (2016) observed that exposure to raphidophytes (e.g., *H. akashiwo*) affected only the motile stages in the development of the Japanese pearl oyster [15]. However, feeding behavior and physiology of adult ribbed mussel, *Geukensia demissa* (61–83 mm shell length), can be affected by *H. akashiwo* [35]. These disparate findings suggest that there may be species-specific differences in the response to *H. akashiwo* exposure [36]. The early-life developmental stages of bivalves may be more sensitive to HABs than adult individuals. In addition, the effects of culture condition on toxin production and interstrain differences of *H. akashiwo* should be also considered in future research [37,38]. For instance, Fredrickson et al. (2011) observed that there are interstrain differences in *H. akashiwo* toxicity. Among four Pacific Northwest *H. akashiwo* strain, only strain 07B was not toxic to ciliate *Strombidinopsis acuminatum* [38].

Several studies have used cellular and molecular biomarkers to explore the effects of HAB species on fish or shellfish [17,39]. For instance, two cellular biomarkers, namely, lysosomal destabilization and p-glycoprotein, were used to examine the effects of *H. akashiwo* on the southeastern oyster *C. virginica* [14]. Here, we also used specific parameters (SOD and T-AOC activity, MDA content) to assess the response of *M. meretrix* to *H. akashiwo* exposure. However, no significant changes in antioxidant activity were observed in clams after exposure to *H. akashiwo*, indicating that *H. akashiwo* did not induce oxidative stress in the clams. Additionally, we comprehensively explored the effects of *H. akashiwo* on clams through transcriptomic analysis as this can significantly provide more information. Our results identified 544 unigenes that were differentially expressed between the control and *H. akashiwo*-treated clams. Apart from genes involved in the lysosomal pathway, one unigene encoding the sodium/glucose cotransporter 2 (SGLT2), belonging to solute carrier family 5, was found to be highly expressed after *H. akashiwo* exposure for 48 h (Figure 4). Consistent with our results, Hanquet et al. (2011) found SGLT expression in Pacific oysters was significantly up-regulated when food was plentiful [40]. Similar to *SGLT2*, the monocarboxylate transporter 12 (MCT12), which belongs to a family of proton-linked plasma membrane transporters that catalyze the transport of monocarboxylates, such as creatine across biological membranes, was significantly up-regulated after 48 h of exposure to *H. akashiwo* (Figure 4). Interestingly, one unigene coding for the molluscan insulin-related peptide 3 (ILP3) was also up-regulated in the *H. akashiwo*-exposed clams. In invertebrates, including mollusks, insulin-related peptide is documented to be important for regulating animal growth [41]. These results suggest that *M. meretrix* may be adapted to digest *H. akashiwo* through the regulation of target genes.

It is widely accepted that phytoplankton biomass can be affected by predators (top-down grazing), including copepods and bivalves. The use of bivalves to control HAB has also been proposed [42]. For instance, Galimany et al. (2020) found that algal bloom species (e.g., *Aureoumbra lagunensis*) can be assimilated by bivalves (e.g., *C. virginica*) [43]. Furthermore, these authors demonstrated that the clearance rates differed among the three examined bivalves and the brown tide-forming alga *A. lagunensis*. Overall, our study and previous reports have demonstrated that bivalves can feed effectively on HAB species, including *H. akashiwo* [34,35]. Further research is required for the investigation of the clearance rates of *H. akashiwo* by filter feeders.

5. Conclusions

Here, we examined the physiological and transcriptomic responses of *M. meretrix* to *H. akashiwo*. Based on our analysis, the *M. meretrix* can feed off *H. akashiwo*, and three physiological and biochemical parameters of clams, were not affected by these algae. The results of RNA-seq showed that the upregulated expression of related genes (e.g., lysosome-related genes) probably facilitates the digestion of *H. akashiwo* by clams. These results indicate that the intensification of shellfish farming and the maintenance of the community diversities of filter feeds may be significant in the control of HABs in coastal ecosystems and farming areas.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes8020067/s1, Figure S1: The 20 highest-ranking KEGG pathways associated with *M. meretrix* DEGs under different conditions: (a) up-regulated genes in the treatment group; (b) down-regulated genes in the treatment group; Figure S2: Photographs of the dissected clams: (a) control; (b) treatment; Table S1: A summary of the transcriptome sequence data; Table S2: Detailed information on 57 core genes expressed under different treatments; Table S3: Analysis of expression change of six selected target genes after normalized against a set of two reference genes.

Author Contributions: Conceptualization, N.J., M.X., X.S. and Y.C.; methodology, N.J., J.L., S.L. and Y.C.; investigation, J.W., S.L., X.Y. and Y.C.; writing—original draft preparation, N.J.; writing—review and editing, N.J., X.S. and Y.C.; supervision, N.J.; project administration, X.S.; funding acquisition, N.J. and X.S. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Ethical review and approval were waived for this study due to it did not involve any endangered or protected species. In addition, the *Meretrix meretrix* is bivalve and member of the invertebrate mollusk family. It differs from other mollusks, such as octopus and squid, for its evolutionary simplicity. In this study, all animal experiments were conducted according to the Animal Care Committee of Jiangsu Ocean University.

Data Availability Statement: In this study, all data generated are included in this article and supplementary material files of it. Further enquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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