Aquatic Toxicology 194 (2018) 121–131

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

# Digital gene expression analysis in the gills of *Ruditapes philippinarum* exposed to short- and long-term exposures of ammonia nitrogen

Ming Cong[a], Huifeng Wu[a,b,*], Tengfei Cao[c], Jiasen Lv[d], Qing Wang[a], Chenglong Ji[a], Chenghua Li[c], Jianmin Zhao[a,*]

[a] Key Laboratory of Coastal Environmental Processes and Ecological Remediation, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai 264003, PR China
[b] Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266237, PR China
[c] School of Marine Sciences, Ningbo University, Ningbo 315211, PR China
[d] Biology School of Yantai University, Yantai 264005, PR China

ARTICLE INFO

*Keywords:*
*Ruditapes philippinarum*
Digital gene expression
Transcriptome
Ammonia nitrogen
Toxicity

ABSTRACT

Previous study revealed severe toxic effects of ammonia nitrogen on *Ruditapes philippinarum* including lysosomal instability, disturbed metabolic profiles, gill tissues with damaged structure, and variation of neurotransmitter concentrations. However, the underlying molecular mechanism was not fully understood yet. In the present study, digital gene expression technology (DGE) was applied to globally screen the key genes and pathways involved in the responses to short- and long-term exposures of ammonia nitrogen. Results of DGE analysis indicated that short-term duration of ammonia exposure affected pathways in Dorso-ventral axis formation, Notch signaling, thyroid hormone signaling and protein processing in endoplasmic reticulum. The long-term exposure led to DEGs significantly enriched in gap junction, immunity, signal and hormone transduction, as well as key substance metabolism pathways. Functional research of significantly changed DEGs suggested that the immunity of *R. philippinarum* was weakened heavily by toxic effects of ammonia nitrogen, as well as neurotransduction and metabolism of important substances. Taken together, the present study provides a molecular support for the previous results of the detrimental toxicity of ammonia exposure in *R. philippinarum*, further work will be performed to investigate the specific genes and their certain functions involved in ammonia toxicity to molluscs.

## 1. Introduction

Over the past decades, ammonia nitrogen has been a consistent pollutant in some marine fishery waters according to the annual reports on the state of Chinese marine environment. Of the two existing forms of ammonia in seawater, the un-ionized form ($NH_3$-N) is more toxic than the ion form ($NH_4^+$) because of its capacity to diffuse through cell membranes (Emmerson et al., 1975). The un-ionized ammonia nitrogen (UIA) can induce many adverse effects in fishes, crustaceans and molluscs, including severe histological changes, neurological dysfunction, growth restriction, respiration impairment and fecundity decrement (Armstrong et al., 2012; Keppler, 2007; Maas et al., 2012; Randall and Tsui, 2002; Smart, 1978). Although the invertebrates (such as molluscs) were generally found to be more tolerant to ammonia stress compared with the vertebrate (Arthur et al., 1987), toxicology studies indicated that ammonia nitrogen caused severe toxicity to the clams. Therefore, it is necessary to clarify the toxic mechanism of ammonia nitrogen on the molluscs in order to provide reliable data for practical treatment of reducing ammonia nitrogen pollution and environmental protection in shellfish aquaculture around the coastal area.

The toxic mechanism of ammonia has been studied more clearly in fishes than other aquatic organisms. It was found that ammonia depolarizes the neurons and activates the N-Methyl-D-aspartic acid (NMDA)-type glutamate receptor, leading to subsequent cell apoptosis in the

*Abbreviations:* DGE, Digital gene expression technology; UIA, un-ionized ammonia nitrogen; NMDA, N-Methyl-D-aspartic acid; DEG, differentially expressed genes; Nr, NCBI non-redundant protein sequences; Nt, NCBI non-redundant nucleotide sequences; Pfam, Protein family; KOG/COG, Clusters of Orthologous Groups of proteins; KOG, KEGG Ortholog database; GO, Gene Ontology; qRT-PCR, quantitative real-time PCR; 18S, 18S rDNA; Actin, beta actin; Tub, beta tubulin; EGF1α, elongation factor 1 alpha; Ubi, ubiquitin; Cyclo, cyclophilin A; ABC, ATP-binding cassette; MRP, multidrug resistance protein; ER, endoplasmic reticulum; MCD, Malonyl-CoA decarboxylase; IFI44L, Interferon-induced protein 44-like; A2M, Alpha-2 macroglobulin; EGF-like, epiderma-growth factor-like; GS, Glutamine synthetase; ABAT, 4-aminobutyrate aminotransferase

* Corresponding authors at: Key Laboratory of Coastal Environmental Processes and Ecological Remediation, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai 264003, PR China.
*E-mail addresses:* hfwu@yic.ac.cn (H. Wu), jmzhao@yic.ac.cn (J. Zhao).

https://doi.org/10.1016/j.aquatox.2017.11.012
Received 25 August 2017; Received in revised form 21 November 2017; Accepted 22 November 2017
Available online 23 November 2017
0166-445X/ 

brain by causing influxes of excessive $Ca^{2+}$ and $K^{+}$ (Randall and Tsui, 2002). However, the related study on molluscs is still in a primary stage. Such toxic studies basically focus on the changes of survival rates, energy

allocation, cellular and immune parameters after ammonia nitrogen exposure (Maas et al., 2012; Keppler, 2007; Wang et al., 2012; Widman et al., 2008). The underlying mechanism of ammonia toxicity in mollusc was not fully elucidated yet. In previous studies, we found that ammonia nitrogen exposure caused a series of adverse effects to the clam *Ruditapes philippinarum*, including decrease in lysosomal stability, disturbance of metabolic profiles, damage to the gill structure and variation of neurotransmitter concentrations (Cong et al., 2017). However, which genes and how many pathways were involved in the toxic reaction of *R. philippinarum* to ammonia nitrogen challenge? These questions were not answered clearly yet. In the present study, the expression profiles of related genes were detected by digital gene expression (DGE) analysis to preliminarily describe the genetic network of the *R. Philippinarum* clams to the ammonia exposure.

DGE is a kind of analysis approach widely used today to explore differentially expressed genes (DEGs) when the target organisms or their tissues are under a particular stress, with or without the background genome information. It has been quickly used in quite a few of aquatic organisms to detect their responses to external factors at gene level, such as *Oryzias melastigma* (Huang et al., 2012), *Crassostrea gigas* (Zhao et al., 2012) and *Chlamys farreri* (Hu et al., 2015). *R. philippinarum* is an important marine mollusc, distributed widely along the Chinese coastal area. As a sedentary habitant, *R. philippinarum* is more inclined to the environment pollutants including ammonia nitrogen. Among *R. philippinarum*'s organs, gill is the first one to contact with the environment and probably is the first target of external pollutants. In the present study, *R. philippinarum* received ammonia exposures for 1 and 30 days respectively to find out the differentially expressed genes involved in acute and subacute toxicities of ammonia nitrogen. Gill tissues were used to analyze the gene expression profiles by using DGE technology, and validated by quantitative real-time PCR to identify the genes and pathways which were involved in the molecular reaction of clams against ammonia nitrogen exposure.

## 2. Materials and methods

### 2.1. Animals and ammonia-N exposure experiments

Healthy clam *R. philippinarum* (averaging 3.56 ± 0.35 cm in shell length) were collected from Yangma Island (with an average background $NH_3$—N concentration of 0.0016 mg/L), Yantai city. The clams were acclimated for 15 days in aerated and filtered seawater (19 ~ 20 °C, pH 8.0, 32 psu), and fed with a mixture of *Isochrysis galbana* and *Chlorella vulgaris Beij*. The seawater in each tank was changed twice every day just before and 2-h after the feeding. Stock solution (1 mol/L) of high purity $NH_4Cl$ was used as the source of the total ammonia. After the acclimation period, all of the clams were randomly divided into two groups as blank (0 mg/L) and ammonia nitrogen (0.1 mg/L) exposure groups with three replicate tanks respectively.

According to the annual reports on the environmental state of China, the actual concentration of ammonia nitrogen in the neritic China seas ranged from 0 to 19.32 mg/L with an average value of 1.82 mg/L. Based on the result of lysosomal destabilization which was an indicator of cellular damage (Keppler, 2007; Aguirre-Martínez et al., 2013), our previous study suggested that 1-day exposure of 0.1 mg/L ammonia nitrogen could cause toxic effects to the calms, and induced significant changes in neurotransmitter concentrations, lysosomal stabilization and metabolite concentrations (Cong et al., 2017). And a 30-day exposure of 0.1 mg/L ammonia nitrogen didn't cause massive mortality to the clams (data not shown). So 0.1 mg/L ammonia nitrogen was used in the present study to evaluate toxic reactions in clam *R. philippinarum* in an ammonia nitrogen polluted environment, and 1-day and 30-day durations of ammonia nitrogen were used to compare the effects of acute and subacute ammonia exposure in clams, respectively. During the 30-days' exposure experiment, gill tissues of the clams were collected into RNAlater® Stabilization Solution (Thermo Fisher) at the 0, 1st and 30th-day, and used as Blank (B), A1, A30 group samples respectively. In order to diminish the individual difference among the same group, five individuals were pooled as one sample. And three samples were prepared in each group at each sampling time-point. The transcriptome samples in the blank, 1-day and 30-day exposure groups were designated as RpB, RpA1 and RpA30 respectively. In addition, another five individual clams from each group were collected as five samples to be used in the real-time RT-PCR experiment.

### 2.2. Total RNA extraction and DGE sequencing

RNA extraction from each sample was carried out following the manual instructions of the Trizol reagent. The purity, concentration and integrity of RNA samples were checked by using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA), Qubit® RNA Assay Kit in Qubit® Library preparation for Transcriptome sequencing 2.0 Flurometer (Life Technologies, CA, USA) and RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA), respectively. A total amount of 3 μg RNA ($A_{260}/A_{280}$ = 1.8) per sample was used as input material for further analysis. Sequencing libraries were generated using NEB Next® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations, and library quality was assessed on the Agilent Bioanalyzer 2100 system. Then the clustered library was sequenced on an Illumina Hiseq 2500 platform and 125 bp/50 bp paired/single-end reads were generated as raw data in fastq format.

### 2.3. Bioinformatics analyses and in silico gene expression analyses

Clean data were got by removing reads containing adapter, ploy-N and other low quality reads from raw data. Q20, Q30, GC-content and sequence duplication level of the clean data were all calculated. After that, transcriptome was assembled by pooling the clean data with high quality from all treatments together. Then gene functions were annotated based on several databases, including Nr (NCBI non-redundant protein sequences), Nt (NCBI non-redundant nucleotide sequences), Pfam (Protein family), KOG/COG (Clusters of Orthologous Groups of proteins), Swiss-Prot (A manually annotated and reviewed protein sequence database), KOG (KEGG Ortholog database) and GO (Gene Ontology). SNP calling was performed by GATK2 software with distance > 5.

Gene expression levels were estimated by RSEM (Li and Dewey, 2011) for each sample. Clean data were mapped back onto the assembled transcriptome and readcount for each gene was obtained from the mapping results. Differential expression analyses of the blank and the ammonia-nitrogen exposed groups were performed by the DESeq R package (1.10.1). The resulting *P* values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted *P* value < 0.05 found by DESeq were assigned as differentially expressed. In addition, a pheatmap was constructed according to the relative expression levels of the differentially expressed genes among the blank, 1-day and 30-day exposure groups (designated as RpB, RpA1 and RpA30, respectively).

### 2.4. GO and KEGG analysis of the DGEs

GO enrichment analysis of the DEGs was implemented by the GOseqR packages based on Wallenius non-central hyper-geometric distribution (Young et al., 2010), which can adjust for gene length bias in DEGs. KEGG (Kanehisa et al., 2008) pathway analysis was performed by KOBAS (Mao et al., 2005) software to enrich the differential expression genes. In addition, interaction between proteins from the DEG