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Genes are Differentially Expressed at Transcriptional Level of *Neocaridina denticulata* Following Short-Term Exposure to Nonylphenol

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Abstract To assess the toxicity of nonylphenol towards aquatic crustaceans, *Neocaridina denticulata* were exposed short-term to sublethal concentration (0.001–0.5 mg/L). Following treatment, differentially expressed genes were identified using suppression subtractive hybridization on samples prepared from whole specimens. There were 20 differentially expressed sequence tags that corresponded to known genes and could be divided into six functional classes: defence, translation, metabolism, ribosomal gene expression, respiration, and genes involved in the stress response. Using semi-quantitative RT–PCR, we found that 14 of the differentially expressed sequence tags significantly responded to nonylphenol, including six at a nominal concentration of 0.01 mg/L; among them, 12 genes were down-regulated. These results suggest that under non-lethal concentrations of nonylphenol, the polluted aquatic environment may still present a potential risk to *N. denticulata*.

Keywords Nonylphenol · *Neocaridina denticulata* · Aquatic crustacean · Suppression subtractive hybridization (SSH) · Expressed sequence tag (EST)

Nonylphenol is a major metabolic product that is formed from the microbial breakdown of alkylphenol ethoxylates (APEs) (Ahel et al. 1996), which are non-ionic surfactants used in a variety of industrial, household, and commercial products and have accumulated in a wide range of marine and aquatic organisms (Ahel et al. 1993; Staples et al. 1998). Nonylphenol primarily travels into the aquatic environment through industrial and municipal wastewater discharges and has been reported in rivers, lakes, and coastal waters around the world. In Taiwan, the deficiency in municipal wastewater treatment systems results in significantly higher concentrations of APE metabolic residues in the local rivers compared to rivers in other countries (Ding et al. 1999). While previous studies have focused on the estrogenic activity of nonylphenol that causes endocrine disruption in many animals, it can also interfere with non-estrogenic activities (Soares et al. 2008). In invertebrates, some studies have demonstrated that nonylphenol can reduce the population growth rate of the marine copepod *Tisbe battagliai* (Bechmann 1999) and the oyster *Crassostrea gigas* (Nice et al. 2000). It has also been observed to damage haemocytes and decrease the cellular immunity of the economic prawn *Macrobrachium rosenbergii* (Sung and Su 2005). Although concerns about the environmental toxicity and pollution of nonylphenol are obvious, field investigations on the effects of nonylphenol on other physiological functions of aquatic organisms are rare.

Many invertebrate toxicity test protocols are routinely used in regulatory toxicity testing, and freshwater crustaceans are currently employed to monitor environmental pollution status because they are a major invertebrate component in most aquatic ecosystems; in addition, their populations are often numerous, and they are easily cultured in the laboratory (Kirkpatrick et al. 2006). *Neocaridina denticulata,* (De Haan, 1844, Crustacea, Decapoda) is not only distributed in rivers throughout eastern Asia and the Hawaiian islands but is also a common shrimp in the fresh waters of Taiwan. Several characteristics of *N. denticulata* make this shrimp a good aquatic indicator for assessing environmental pollution: small size (2–3 cm), spontaneous interbreeding, and the absence of a metamorphosis stage

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during development. Since 2005, the Environmental Protection Administration of Taiwan announced that this organism was to be used as an indicator in acute toxicity assays to assess the quality of various water bodies or effluents from industrial districts.

Our previous study demonstrated that nonylphenol could have a stimulatory effect on the immune response of prawns at a low concentration; however, increased concentrations of nonylphenol may damage prawns and subsequently increase their susceptibility to pathogens (Sung and Ye 2009). Therefore, one must determine what effect nonylphenol has on the global physiology of *N. denticulata*, and whether the increased susceptibility is caused by the immune system or whether other physiological functions are affected by nonylphenol. Therefore, the aim of this study was to determine the expression of a comprehensive set of genes of *N. denticulata* after exposure to nonylphenol. The expressed sequence tags (ESTs) in nonylphenol-treated and untreated shrimps were isolated and identified through suppression subtractive hybridization (SSH). To assess the regulation of the differential gene expression induced by nonylphenol, messenger RNA expression of selected genes associated with various physiological functions were analyzed. The results from this study will be useful in assessing the feasibility of employing *N. denticulata* as an aquatic indicator, and whether the ESTs that have been found to be differentially expression can serve as potential genomic biomarkers for the biomonitoring of hidden risks caused by pollutants in the aquatic environment for aquatic organisms.

Materials and Methods

Shrimp (*N. denticulata*) with body lengths of 2–3 cm/individual were acclimated in 20-L glass aquaria containing fresh pond water and water plants (*Egeria densa* Planch) at 28°C, pH 6.9–7.5, oxygen concentration >4 mg/L and a 12-h light–dark photoperiod. Prior to the experiments, the shrimp were acclimated for at least one week, and the expression of the prophenoloxidase gene (*propo*) was used as a parameter to determine whether each batch of shrimp was usable. They were considered usable only when the mRNA ratio of *propo* to *actin* (as an internal control) was 0.1–0.3. The shrimp were fed mosquito larvae (also called blood worms) twice a day. The stocking densities were maintained at 100 individuals per aquarium.

Nonylphenol (Chem Service Co., S-306) was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 1×10^5 mg/L as a stock solution and stored at room temperature. Prior to immersion, the stock solution was serially diluted with fresh pond water to different concentrations. Preliminary tests had showed that the mortality of shrimp continuously treated with nonylphenol concentrations of 0.1, 0.5, 0.75 and 1 mg/L for 1 day were 0, 0, 13 and 35%, respectively; the mortality of the solvent control (DMSO-treated group) was 0%. Therefore, for different experiments, shrimp were continuously treated with different concentrations (0.001, 0.01, 0.1 and 0.5 mg/L) of nonylphenol at 60 individuals in 20-L glass aquaria containing 18 L fresh pond water for 1 day. The solvent control shrimp were treated with pond water containing a 10^4-fold dilution of DMSO.

Because the size of the shrimp is too small to sufficiently collect different tissues for different experiments, a tissue lysate supernatant (TLS) was prepared from at least ten whole individuals. Briefly, shrimps were immersed in ice-cold PBS for 1–2 min and ground into a powder using a liquid nitrogen-chilled mortar with a Teflon pestle (Kontes, Vineland, NJ, USA). The tissue homogenate was then used directly to isolate RNA. One tissue homogenate sample was prepared from ten individuals. For RNA isolation, 50–100 mg of the tissue homogenate was resuspended in 1 mL of TRIzol reagent (Invitrogen, USA) and incubated at room temperature for 5 min. The solution was mixed vigorously for 15 s with 0.2 mL of chloroform and then left at room temperature for 10 min. After the mixture was centrifuged at 12,000 × g for 15 min at 4°C, the uppermost layer was collected and mixed with 0.5 mL of isopropanol at room temperature for 10 min. Following centrifugation, the RNA pellet was desalted by 75% (v/v) ethanol prepared with diethylpyrocarbonate (DEPC; Sigma)-treated sterile water and dissolved in 10 μL of DEPC-treated water. PolyA^+ RNA was purified from this preparation with a Dynabeads mRNA Purification Kit (Invitrogen). Prior to the experiments, the concentration of RNA was quantified by measuring the absorbance of the RNA solution at wavelengths of 260–280 nm.

SSH was performed to generate a subtracted cDNA library from RNA isolated from both NP-treated shrimp and solvent control shrimp as described by Lu et al. (2009) using a Clontech PCR-Select™ cDNA Subtraction Kit (Clontech). For the positive subtraction, the two adaptor-ligated tester cDNA samples (NP-treated samples) were separately mixed with driver cDNA (solvent control) so that the concentration ratio of tester to driver was 1:6. For the negative subtraction, the tester and driver cDNA samples were prepared from solvent control and NP-treated samples, respectively. The efficiency of subtraction was evaluated by PCR performed on the tester (un-subtracted) and subtracted cDNAs for 25 cycles with forward and reverse primers against the β-actin gene, which was used as a meaningful internal control. The β-actin primers were Actin-F (5′-CCCAG AGCAAGA GAGGTA-3′) and Actin-R (5′-GCGTATCCT TCGTAGATGGG-3′), which yielded a 309-bp fragment (HO762521). The PCR products were ligated into a TA

plasmid to construct the subtractive libraries. To determine the genetic constitution of the inserts in the subtractive libraries, the libraries were further classified by their restriction enzyme digestion patterns; different inserts were sequenced by the Sequencing Core Lab of the National Yang-Ming University Genome Research Centre (YMGC). The following software was used for DNA sequence analyses and comparison: DNAStar for sequence alignment, BLASTN for nucleotide sequence comparison and BLASTX for amino acid sequence comparison. For each comparison, an *e* value of less than 10^{-6} was regarded as a positive result. Databases from the National Center for Biotechnology Information (NCBI), European Bioinformatics Institute (EBI) and UCSC Genome Bioinformatics website were used for comparison.

After treatment with different concentrations of nonylphenol, the differential gene expression pattern was verified by semi-quantitative RT–PCR against total RNA (0.25 μg cDNA). In this study, primers for the genes isolated by subtraction were designed and used in PCR. In addition, β-actin mRNA expression was used as a relevant internal control for these experiments. Data shown in the figures from this study are expressed as a ratio of control, which was defined as the ratio of one target gene from the nonylphenol-treated group divided by the ratio of the same target gene from the control group. Because outbred animals were used as samples in this study, the phenotypic background was most likely significantly heterogeneous among individual animals. The means of the average relative values of at least five replicates from 50 individuals were statistically analysed to examine the effect of nonylphenol on TLS mRNA expression using ANOVA and Duncan's multiple range tests, with a specified significance level of either $P < 0.05$ or $P < 0.01$.

Results and Discussion

To better understand the global changes in gene expression patterns of *N. denticulata* after treatment with nonylphenol, the ESTs from shrimp treated with a sublethal concentration (0.5 mg/L) of nonylphenol for one day were isolated from the forward and reverse subtractive libraries. In total, 107 ESTs were isolated from 425 clones and sequenced. After removing duplicates, there were 65 unique ESTs. Among those, 20 ESTs were identified with arthropod sequences of known or hypothetical function in GenBank with an *e*-value of less than 10^{-20} and classified into six different types according to their physiological function, including defence-related genes (NP1, FL640907; NP2, FL640908; NP3, FL640909; NP4, FL640911; NP13, FL640922; NP15, FL640931), translation-related genes (NP5, NP6), respiration-related genes (NP7, FL640914; NP11, FL640918; NP12, FL640920; NP17, FL640933; NP19, FL640935), metabolism-related genes (NP8, FL640915; NP10, FL640917; NP16, FL640932), ribosomal genes (NP9, FL640916; NP20, FL640939) and stress-related genes(NP14, FL640930; NP18, FL640934). The other 45 ESTs could not be identified because the *e*-value was greater than 10^{-20}. The results found that only 31% of identified ESTs corresponded to genes of known or hypothetical function, and the function of 69% of the ESTs were unknown. Moens et al. (2007) found that 53.2% of all target genes regulated by nonylphenol in the liver of the common carp (*Cyprinus carpio*) were functionally unknown. In fact, with the exception of a few organisms (e.g., human, zebrafish, drosophila, and nematode) whose genomes have been completely sequenced and characterized, a large number of genes have not been identified. Therefore, to clarify the effect of nonylphenol on the physiological functions of *N. denticulata*, it is not only necessary to characterize the unknown genes responding to nonylphenol but also to develop a genomic analysis system that involves a greater number of ESTs.

Representatives from some of the genes of known function and unknown function, including 11 functional genes and 12 unidentified genes, for which specific primers could be designed were selected for further analysis using semi-quantitative PCR on the cDNA from the same samples used for the SSH analysis. The results showed that 14 (five known and nine unknown) genes out of the 23 target genes were significantly different between the nonylphenol-treated group and the control group; among them, only two genes (NP18, referred to as *trehalose-6-phophate synthase, tps*, and uNP2) were upregulated and the remainder were downregulated, including four known genes: NP2, NP6, NP8 and NP16, which are known as *hemocyanin (hc)*, *elongation factor-1 alpha (ef-1α)*, *cathepsin-L like cysteine protease* (*catl*) and *inosine monophosphate dehydrogenase 1* (*impdh-1*), respectively. In the liver of carp exposed to nonylphenol, 64% of known target genes were involved in the defence response and in respiratory and metabolic processes (Moens et al. 2007). In this study, 70% of known target genes (14/20) that responded to nonylphenol were also classified into those three functional groups. Of the selected genes, most genes (86% of them) were downregulated by nonylphenol, including the following four known genes: defence-related *hc* (Decker and Rimke 1998), translation-involved *ef-1α*, metabolism-related *impdh-1*, and *catl*, which may play a role in either metabolism or defence (Ma et al. 2010). For many crustaceans, the environmental stress provoked by pollutants appears to be an important factor in reducing immunocompetence and is signalled by the appearance or increased prevalence of disease (Victor et al. 1990). Previous studies have indicated that nonylphenol can modulate

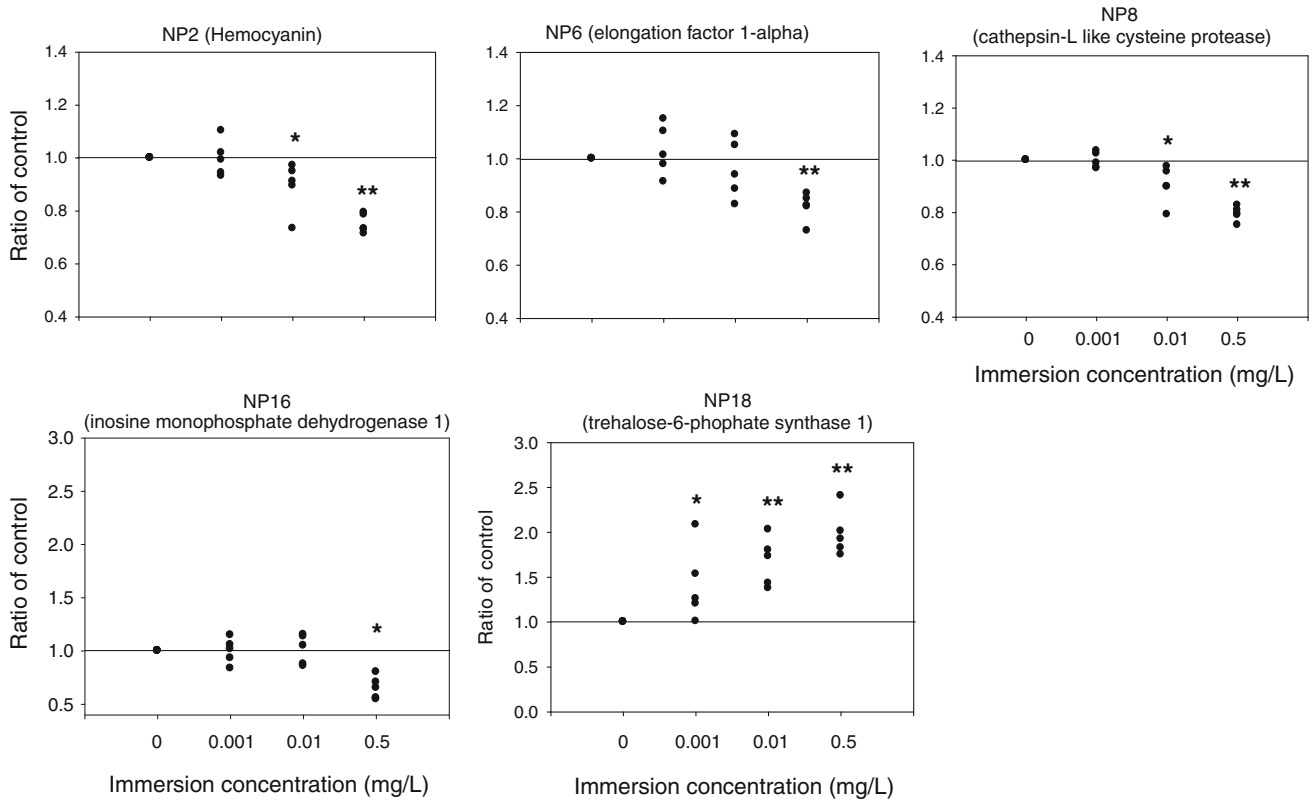

Fig. 1 Messenger RNA expression of function-known genes of *Neocaridina denticulata* treated with various concentrations of nonylphenol (NP) for one day. A semi-quantitative determination of mRNA levels of target genes and an internal control (actin) was carried out. The data are expressed as "ratio of control", which was calculated as the ratio of target gene (x) from NP-treated group/ratio of the target gene (x) from PBS-treated group. Significant differences in mRNA expression between the NP-treated group and the control group are indicated with single asterisk ($P < 0.05$) or double asterisks ($P < 0.01$)

the immune function of the marine bivalve *Mytilus* (Canesi et al. 2007) and increase the susceptibility of the prawn *M. rosenbergii* to infection (Sung and Ye 2009). Our findings that most of the genes concerning defence-related *hc*, translation-involved *ef-1α*, metabolism-related *impdh-1* and metabolism- or defence- related *catl* were downregulated after nonylphenol treatment may explain, in part, the increased susceptibility to disease.

It is worth noting that of all of the known-function genes, only one stress-related gene, *trehalose-6-phosphate synthase* (*tps*), was found to be upregulated by nonylphenol. Trehalose accumulation in the chironomid *Polypedilum vanderplanki* suggests that this gene plays a role in physiological and biochemical adaptations under extreme environmental conditions (Sakurai et al. 2008). Chung (2008) provided evidence of the presence of trehalose in hemocytes and TPS in the tissues of the blue crab (*Callinectes sapidus*) and postulated a functional role for this gene in energy metabolism and physiological adaptation. In this study, the elevation of *tps* expression after nonylphenol exposure appears to support their observation.

To further investigate the genes most sensitive to nonylphenol, the mRNA levels of fourteen genes that were differentially expressed were measured on day 1 after the shrimp were treated with various concentrations of nonylphenol. Compared to the control group, the expression of all target genes was significantly altered after treatment with 0.5 mg/L of nonylphenol. In the 0.01 mg/L-treated group, five of the genes were significantly affected, including two function-known genes, *catl* (NP8) and *tps* (NP18) and three unidentified genes (uNP2, uNP5 and uNP15) (Figs. 1, 2). Only the mRNA level of *tps* was elevated after treatment with 0.001 mg/L of NP (Fig. 1). Moens et al. (2007) found that most genes in the liver of the common carp were affected after 96 h of exposure at the highest concentration of nonylphenol (0.5 mg/L), whereas a 24-h exposure resulted in fewer gene expression changes. This result suggests that an animal testing model developed using *N. denticulata* may be valuable in monitoring pollutants in aquatic environments, and in assessing the general health conditions of freshwater ecosystems.

In conclusion, the sublethal effects of nonylphenol to *N. denticulata* are broad through the changes in multiple genes at the transcriptional level. Although the TPS response of *N. denticulata* related to stress due to the presence of nonylphenol can be induced, the expression of

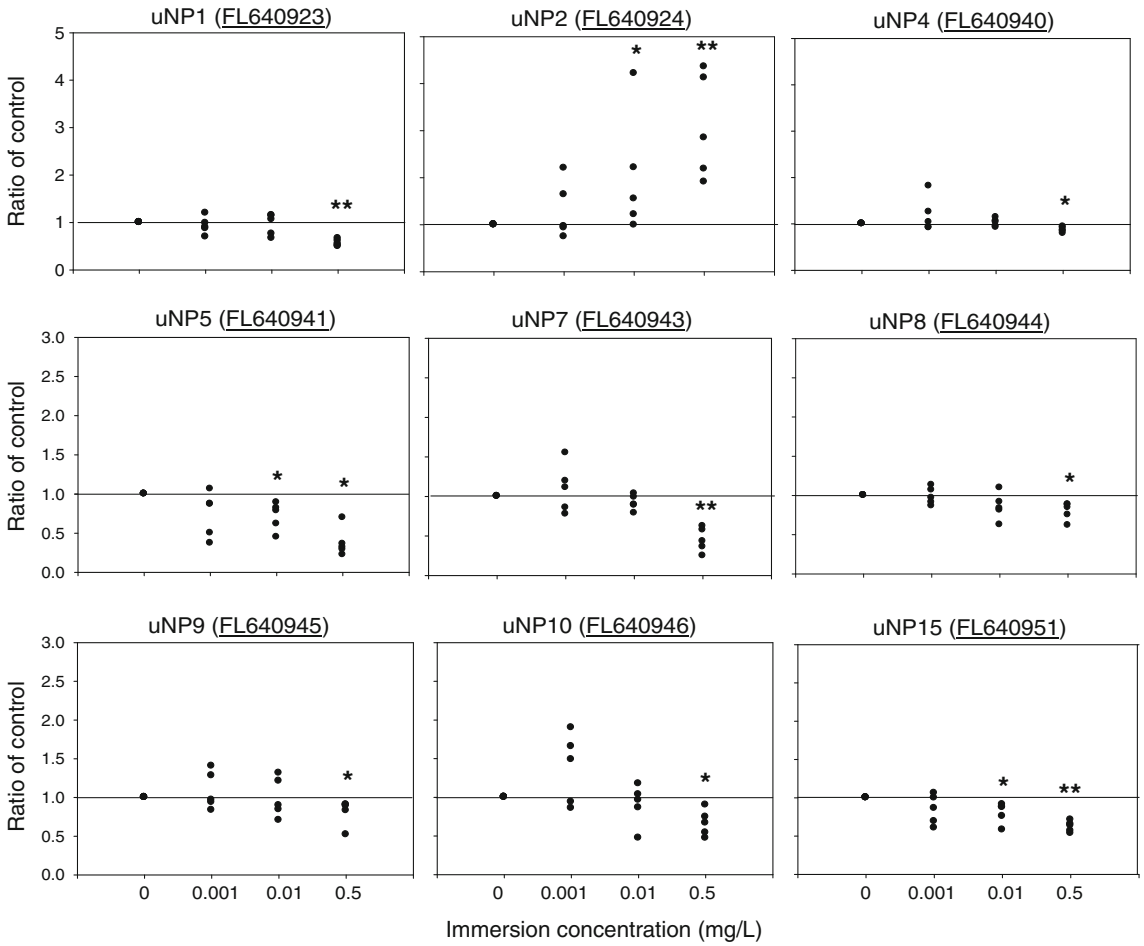

Fig. 2 Messenger RNA expression of unknown genes of *Neocaridina denticulata* treated with various concentrations of nonylphenol (NP) for one day. A semi-quantitative determination of mRNA levels of target genes and an internal control (actin) was carried out. The data are expressed as "ratio of control", which was calculated as the ratio of target gene (x) from NP-treated group/ratio of the target gene (x) from PBS-treated group. uNP denotes ESTs that have not been identified in the public databases and are regarded as function unknown genes

several genes concerning metabolism- and immunity-related functions was downregulated at the RNA level. These results suggest that the ESTs with differential expression derived from *N. denticulata* may be useful as potential biomarkers for the evaluation of environmental pollutants.

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