Ecotoxicology (2019) 28:167–174
https://doi.org/10.1007/s10646-018-2009-2

Evaluation of 4-nonylphenol and bisphenol A toxicity using multiple molecular biomarkers in the water flea *Daphnia magna*

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Accepted: 10 December 2018 / Published online: 5 January 2019
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Abstract

Alkylphenols are well-known endocrine disruptors and may cause developmental and reproductive disorders in aquatic organisms. *Daphnia magna* is commonly used in ecotoxicological studies as a promising model species to investigate the effects of endocrine distruptors. In the present study, transcriptional modulation of eleven potential molecular indicators related to detoxification, antioxidant, development, and cellular stress was analyzed in *D. magna* exposed to different concentrations of bisphenol A (BPA) and 4-nonylphenol (4-NP) for 24 h and 48 h, using real-time qPCR. A hierarchical clustering analysis was applied to investigate relations among molecular markers depending on the compound, exposure duration, and concentration. Our findings suggested that GSH-related systems and stress proteins may be involved in cellular defense against BPA and 4-NP-mediated toxicity with different modes of action. Furthermore, these compounds may interrupt molting and reproduction in daphnids. In particular, *D. magna* GSH-related genes seem to be strongly affected by 4-NP exposure, indicating their potential as molecular biomarkers.

Keywords Biomarkers · Bisphenol A · Daphnia magna · Water flea · 4-nonylphenol

Introduction

Alkylphenols (APs), such as bisphenol A (BPA) and 4-nonylphenol (4-NP), are representative endocrine disruptors (EDs) and have received considerable attention owing to their common occurrence in aquatic environments as a consequence of high manufacturing rates and widespread usage (Sharma et al. 2009). In particular, 4-NP can be bioaccumulated by aquatic organisms and is hard to biodegradation (Soares et al. 2008). APs act as hormone mimics, and their estrogenic effects can elicit early developmental abnormalities and reproductive dysfunctions, such as inhibition of naupliar development, shifting sex ratios, disruption of regenerations, and abnormality of molting and mouthpart structure in aquatic invertebrates (Gross et al. 2001; Watts et al. 2003; Forget-Leray et al. 2005). Due to their detrimental effects, use of bisphenol A and nonylphenol are restricted or banned in European Union (Soares et al. 2008; Grignard et al. 2012).

More recently, it has been reported that BPA and 4-NP can induce oxidative stress in vertebrates (Yazdani et al. 2016) and produce reactive oxygen species (ROS), such as hydroxyl radicals in zebrafish (Wu et al. 2011). Despite harmful effects of BPA and 4-NP, little information is available on APs-mediated oxidative stress responses in aquatic organisms. ROS can be removed by non-enzymatic (e.g. GSH) and enzymatic (e.g. GST, SOD, CAT) antioxidant defense systems. These antioxidants play a key role in maintenance cellular redox balance, thus they are considered to be reliable molecular biomarkers for assessing impacts of chemical-induced oxidative stress in living organisms (Wu et al. 2011).

Biomarkers established in model organisms for various environmental chemicals have been extensively used in the past decades to monitor the health of aquatic ecosystems (Rotchell and Ostrander 2003; Moore et al. 2004; Dagnino et al. 2007). However, the validity of single biomarker in previous studies may be limited by the lack of both sensitivity and specificity. Therefore, in order to understand the effects of EDs on cellular regulation pathways, it is necessary to conduct simultaneous and sequential comparisons

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after different exposure times and in different concentrations, using multiple biomarkers.

Freshwater water flea, *Daphnia magna* is an already established aquatic test species in the field of ecotoxicology (Shaw et al. 2008), and is considered valid indicator of developmental and reproductive disorders induced by EDs (Kang et al. 2014). Reduction of growth and fecundity, alteration of molting behavior, alteration of sex ratios, and genotoxicity by oxidative stress in *D. magna* due to 17b-oestradiol (E_2), BPA, or NP were reported previously (Brennan et al. 2006; Park and Choi 2009). However, molecular effects of EDs are rarely studied in daphnids, despite the availability of a complete genome database (Colbourne et al. 2011).

In the present study, we evaluated eleven molecular markers associated with detoxification, antioxidants, development, and cellular stress in *D. magna* exposed to different BPA and 4-NP concentrations for 24 h and 48 h, respectively. In addition, we created a heat map using a hierarchical clustering analysis to explore the relationships among molecular biomarkers in response to exposure concentration and respective exposure time.

Materials and methods

Culture and maintenance

Daphnia magna were obtained from National Institute of Environmental Research (Incheon, South Korea) and cultured under a 12:12 h light/dark regime at 22 ± 1 °C. Culture media was produced according to the ISO 6341 (ISO 2012) and renewed every second day. Animals were fed daily with *Chlorella vulgaris* ($3.0–3.5 \times 10^{6\text{-}8}$ cells/ml, 1 ml/day).

Chemical exposure

Bisphenol A (BPA, CAS No.80-05-7) and 4-Nonylphenol (4-NP, CAS No.104-40-5) were purchased from Sigma-Aldrich. Neonate *D. magna* (<24 h) produced by adult individuals (2 weeks after hatching) were used as test organisms. For exposure test, stock solutions of BPA (5 mg/ml) and 4-NP (15 µg/ml) were prepared by dimethyl sulfoxide (DMSO). Final concentrations of BPA (0.2, 1 and 5 mg/L) and 4-NP (0.6, 3 and 15 µg/L) were exposed for 24 h and 48 h in neonates of *D. magna* (50 individuals/concentration; 500 ml). The maximum amount of DMSO was 0.01% in all experiments, which is known to not show toxic effects (Barbosa et al. 2003). After exposure, each sample was pooled and used for RNA extraction. Food was not supplied, and media was not changed during exposure to chemicals. Exposure concentrations were determined based on acute toxicity values (Kim et al. 2017).

Table 1 List of qRT-PCR primers used in this study

Gene	GenBank accession no.	Primer sequence (5'–3')
GST delta	AHD24663.1	F: ACAATCGCGGATCTTTCACT R: GCATCGTTCCATCCATTCTT
GST mu	KX358695	F: CATTGATGCCGAGATGTGAAG R: CATTTGTACCGATAAAGTTGTGC
GST sigma	KX358696	F: GGGCGGATTTGGCTTATTATTC R: CCAACATGTGCAACCAACTTC
GST zeta	KX358697	F: CCAGCGTTTCAAAGTGAGG R: CAAACTGTTTAAGCACAGCC
GPx	KX358698	F: TGAAGCAGCTGACCCATAAG R: GCAACTTGGTTTCGTTGAGA
GRx	KX358694	F: ACAATCGCGGATCTTTCACT R: GCATCGTTCCATCCATTCTT
TrxR	KX358699	F: TGGCCAATAATTCCAAACTG R: TCCAATGCATCTGGGTTTC
EcR	AB274824.1	F: CACCACAACCAACTGCATTTA R: CCATTAATGTCAAGATCCCACA
Vtg1	AB114859.1	F: CTCGCATCTCGTCTGATGTT R: GAGAATTGACGTTGCGAAGAG
Hsp70	ACB11340.1	F: GGGTAATCGTACAACACCATCTTA R: CCTTCCACTTCCTTCAGCTTATG
Hsp90	KX358700	F: CCGAGGAAGAGAAACCAAAG R: CGTCGACCGAATACTTCTCC
β-actin	AJ292554.1	F: CCACACTGTCCCCATTTATGAAG R: CGCGACCAGCCAAATCC
ubc	WFes0004602	F: TCACCTGCACTCACCATTTC R: AATCTCCGGAACCAAAGGAT

RNA extraction and cDNA synthesis

D. magna exposed to BPA and 4-NP in different concentrations were respectively homogenized in 5-time volumes of TRIZOL reagent (Thermo Fisher Scientific Inc., Ambion). Total RNA was extracted according to the manufacturer's instructions. cDNA was synthesized from 2 µg of the total RNA using ReverTra Ace® qPCR RT Master Mix (Toyobo Corp., JAPAN).

Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR)

A qRT-PCR reaction including 1 µL of cDNA, 0.2 µM of each primer (Table 1), and PCR premix was performed with a CFX96™ real-time PCR system (Bio-Rad, Hercules, CA, USA). The PCR cycling conditions were as follows: 3 min at 95 °C; 40 cycles of 10 s at 95°C, 30 s at 55°C, and 30 s at 72 °C; 10 min at 72 °C. Agarose gel electrophoresis, melting curve analysis, and sequence analysis were carried out to check the specific PCR product. A SYBR green dye was used for detection of amplicon. *D. magna β-actin* and ubiquitin C (*ubc)* were used as reference genes, and $2^{-\Delta\Delta CT}$

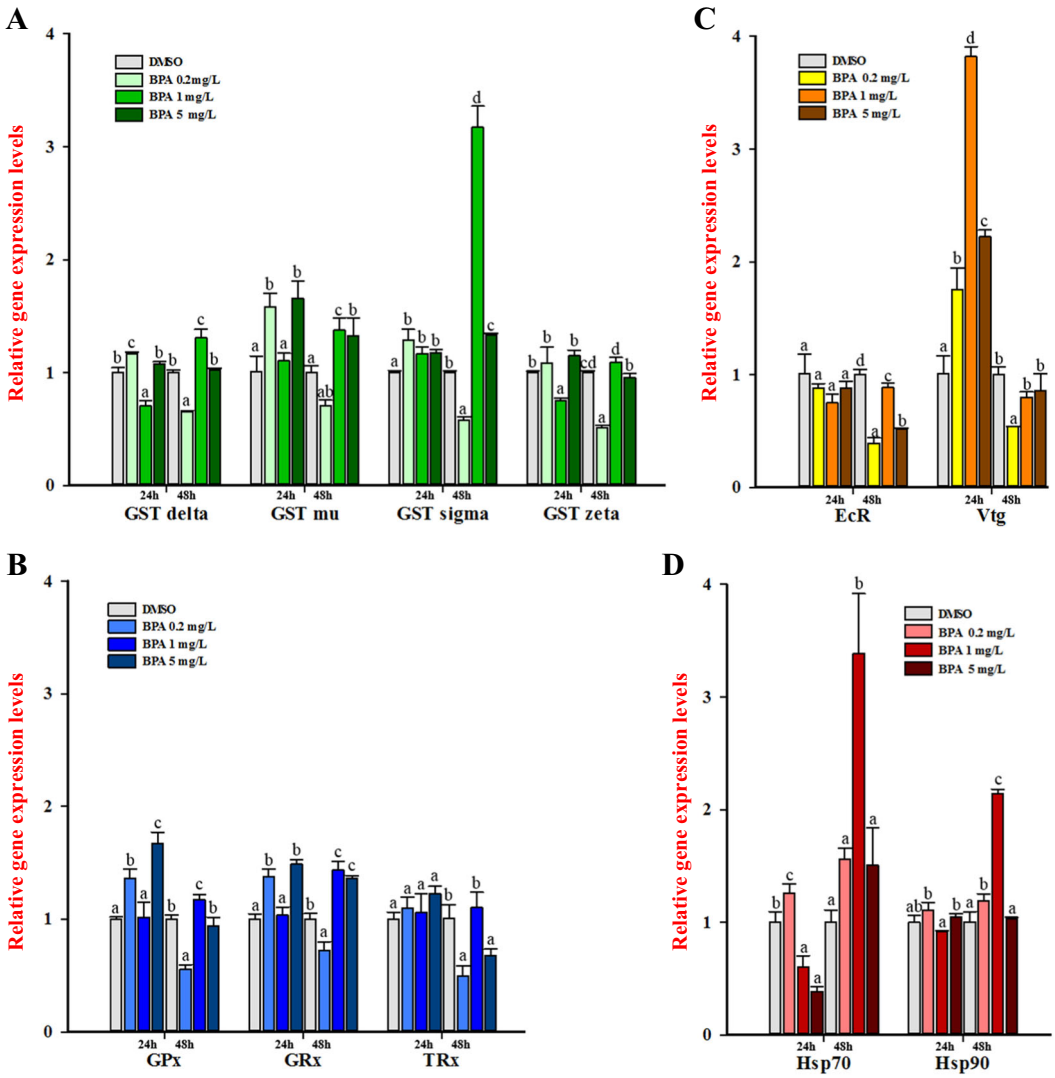

Fig. 1 Transcriptional modulation of eleven genes - related to detoxification **A**, development and reproduction **B**, antioxidant **C**, and stress **D** in Daphnia magna - exposed to BPA (0.2, 1, and 5 mg/L) for 24 h and 48 h, respectively. Data are shown as means ± S.D. of 3 replicates. Different lowercase letters indicate significant differences between concentrations at each time point using one-way ANOVA followed by Turkey's test.

method was applied to determine relative mRNA expression level of target genes (Livak and Schmittgen 2001). All samples were analyzed in triplicates. A heat map was created to show transcript profiles and hierarchical clustering performed by Pearson's correlation using MeV software (ver. 4.9; Dana-Farber Cancer Institute, Boston, MA, USA).

Statistical analysis

All data was presented as means ± standard deviation (SD) of three replicates. Statistical significance of relative mRNA expression between control and experimental groups was analyzed by a one-way analysis of variance (one-way ANOVA; Tukey's test) using PASW statistics ver. 18.0 (SPSS Inc., Chicago, IL, USA).

Results and discussion

Transcriptional modulation of detoxification and antioxidant-related genes

We screened the transcriptional changes of eleven genes associated with detoxification (*GST-delta*, *GST-mu*, *GST-sigma*, and *GST-zeta*), antioxidant (*GRx*, *TRx*, and *GPx*), development and reproduction (*EcR* and *Vtg*), and stress (*Hsp70* and *Hsp90*) in *D. magna* exposed to BPA and 4-NP for 24 h and 48 h, respectively. The GST superfamily is a major group of the phase II detoxification metabolism and comprises various isoforms. Each GST isoform shows different catalytic activity depending on its substrate specificity (Sheehan et al. 2001). In the present study, the

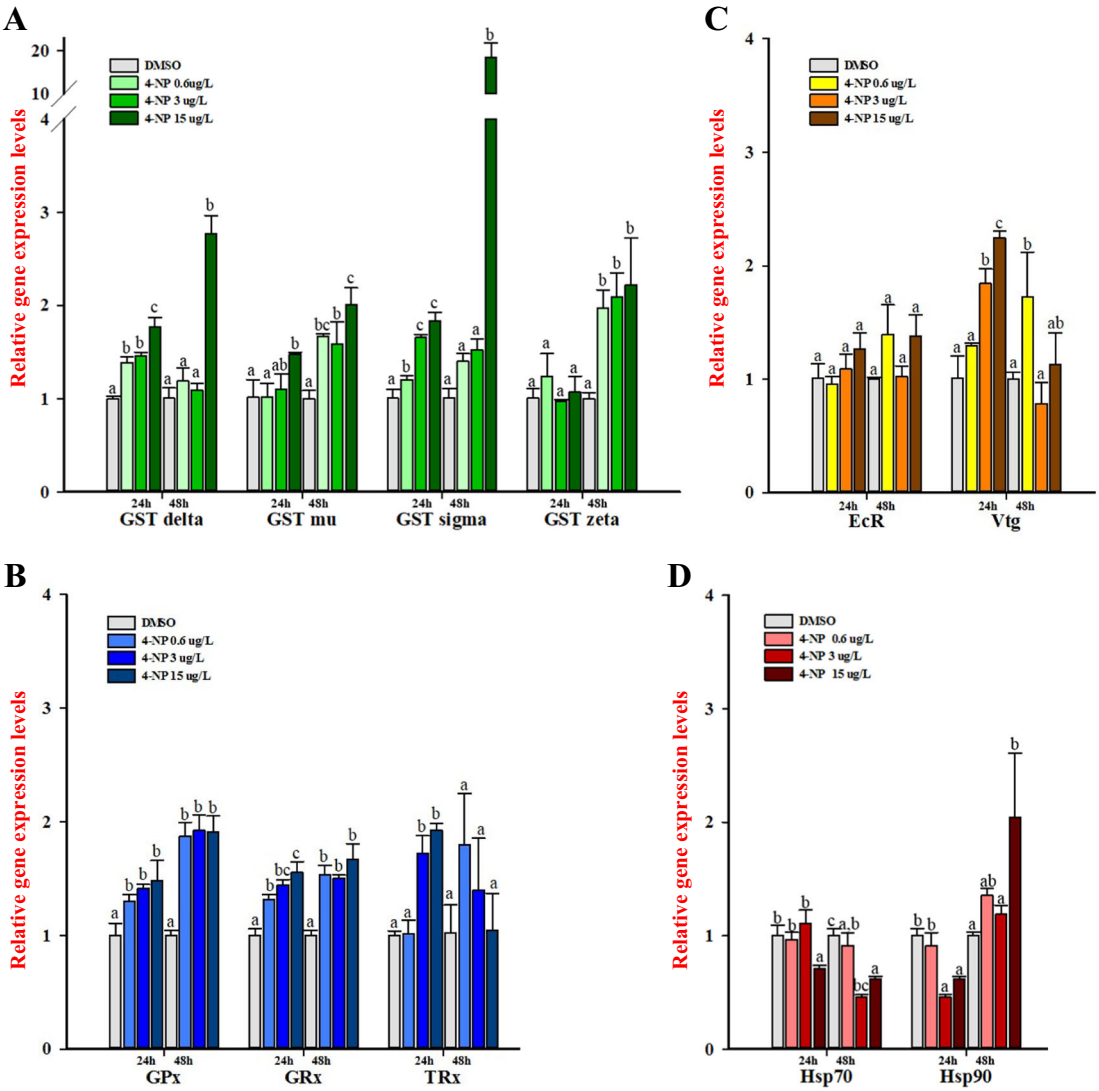

Fig. 2 Transcriptional modulation of eleven genes - related to detoxification **A**, development and reproduction **B**, antioxidant **C**, and stress **D** in Daphnia magna - exposed to 4-NP (0.6, 3 and 15 mg/L)for 24 h and 48 h, respectively. Data are shown as means ± S.D. of 3 replicates. Different lowercase letters indicate significant differences between concentrations at each time point using one-way ANOVA followed by Turkey's test

expression of most GST isoforms was upregulated by BPA (Fig. 1a) and 4-NP (Fig. 2a) at almost all combinations of exposure times and concentrations. In the NP-exposed group, this gene expression showed particular exposure time- and concentration-dependent patterns. Previous studies suggest that 4-NP or BPA may induce oxidative stress and modulate the expression of antioxidants including GSTs as a cellular defense in aquatic organisms (Nair et al. 2011; Kim et al. 2017). Among GST isoforms, *GST-sigma*, which showed the highest level of expression in our study, is known to play a key role in antioxidant defense against environmental compounds, such as 4-NP (Nair et al. 2011).

We also analyzed transcriptional levels of other antioxidants, *GPx*, *GRx*, and *TrxR*, as glutathione and thioredoxin play important roles in the intracellular redox homeostasis (Zitka et al. 2012). As shown in Figs 1b and 2b, *GPx* and *GRx* expression was induced by BPA and 4-NP, whereas *TrxR* was only upregulated by 4-NP. GPx eliminates hydrogen peroxides through oxidation of reduced GSH to GSSG, and GRx is a GSH-dependent thiol-disulphide oxidoreductase which reduces GSH-mixed disulphides (Holmgren et al. 2005; Zitka et al. 2012). Thus, a GSH-dependent redox system may be a vital cellular protective antioxidant in *D. magna* exposed to BPA and 4-NP. Under 4-NP exposure, *TrxR* was significantly upregulated after 24 h in a concentration-dependent manner (Fig. 2b). The Trx system, which comprises NADPH-dependent TrxR and Trx, participates in the removal of hydrogen peroxide (Bhattacharjee et al. 2017). In our previous study, the expression of *Cu/Zn-SOD*, *Mn-SOD*, and catalase (*CAT*) genes was upregulated after 48-h of NP exposure, and after 24-h of BPA exposure (Kim et al. 2017). These results

suggest that strong SOD activation would increase H_2O_2 levels and eventually trigger the activation of an H_2O_2 degradation system-related genes, such as *GPx* and *TrxR*.

Transcriptional modulation of development and reproduction-related genes

Previous studies reported that BPA and 4-NP cause disruption of growth and reproduction activities in *D. magna* (Brennan et al. 2006; Jeong et al. 2013). Therefore, we measured expression levels of two molecular markers related to developmental and reproduction: *EcR* and *Vtg*. As a result, *EcR* gene expression was not regulated or was slightly downregulated, whereas *Vtg* was strongly expressed after short exposure times to BPA (Fig. 1c) and 4-NP (Fig. 2c). Ecdysone (Ec) plays an essential role for development, molting, and metamorphosis in arthropods (Kato et al. 2007). Ec binds to the ecdysone receptor (EcR) and induces transcription of target genes by forming a heterodimer with the ultraspiracle (USP) (Yao et al. 1993). It is known that BPA acts an anti-ecdysteroid in daphnids, leading to retardation in molting and naupliar development (Mu et al. 2005). In *D. magna* exposed to BPA for 48 h, adverse effects on molting process manifested due to the inhibition of cuticle protein expression (Jeong et al. 2013). Hwang et al. (2010) also demonstrated that the transcriptional level of *EcR* in *Tigriopus japonicus* was reduced following BPA exposure. In contrast, the midge *Chironomus riparius* showed upregulation of *EcR* in response to BPA exposure for less than 24 h (Planelló et al. 2008).

Regarding *Vtg1* gene, most concentrations of BPA and 4-NP elicited gene expression after a short time of exposure (Fig. 1c and 2c). The induction of vitellogenin (VTG), which is important in development and reproduction, is therefore one of the most common biological indicator to EDs exposure in fish and invertebrates (Puinean and Rotchell 2006; Matozzo et al. 2008). However, it is known that induction of VTG may be estrogen-independent in arthropods (Hannas et al. 2011). Ecdysteroids and JH induce vitellogenesis in insects, and ecdysteroids can stimulate or repress this reaction (Handler and Postlethwait 2005). Two *Vtg* genes, *vtg1* and *vtg2* have been identified in daphnids, and presence of the JH-responsive element (JHRE) and the ecdysteroid-responsive element (EcRE) was confirmed in the promoter of these genes (Tokishita et al. 2006). In *D. magna* exposed to BPA and 4-NP for 72 h, 4-NP was inducer of *vtg2*, whereas BPA had little effect on its expression, as found in a previous study (Hannas et al. 2011). The authors suggested that *vtg2* mRNA was suppressed by ecdysteroids and showed no response to estrogenic chemicals in daphnids. In addition, they demonstrated that transcriptional modulation of *vtg* genes depends on xenobiotics with either ecdysteroidal or anti-ecdysteroidal activity. Genomic expression responses in *D. magna* exposed to BPA for 48 h showed repression of *Vtg* expression (Jeong et al. 2013), which is consistent with our result of downregulated *Vtg* expression in *D. magna* exposed to BPA and 4-NP for 48 h. Taken together, these findings indicate that BPA and 4-NP can modulate EcR-mediated ecdysteroid pathways and vitellogenesis. The observed discrepancy of *EcR* and *Vtg* expression emphasizes the need for further research on the regulation of ecdysteroid hormone pathways in response to EDs.

Transcriptional modulation of stress-related genes

Hsp70 and Hsp90 are prominent stress proteins expressed following cellular perturbation. As shown in Fig. 1d and 2d, *Hsp70* expression in our study was induced by BPA but not altered upon 4-NP exposure for 48 h. Similarly, Rhee et al. (2009) reported different respective responses of *Hsp70* in *T. japonicus* exposed to BPA and 4-NP. Upregulation of the *Hsp70* gene was also found in the midges after exposure to 3 mg/L BPA for 12 h and 24 h and to 100 mg/L NP for 24 h (Planelló et al. 2008).

Regarding *Hsp90* gene, our results show that exposures to 1 mg/L BPA and 15 mg/L 4-NP, respectively, for 48 h elicited significant activation, which was not observed after 24 h of exposure (Fig. 1d, 2d). In the marine crab *Charybdis japonica*, *Hsp90* mRNA expression was significantly upregulated in a dose-dependent manner after exposure to 4-NP and BPA for 96 h (Park and Kwak 2014). These findings suggest that a transcriptional response of *Hsps* following EDs exposure is species-specific and depends on exposure time and concentration (Lauritano et al. 2012). Hsp70 and Hsp90 are also involved in interaction with steroid hormone receptors, indicating that they may be potential targets for EDs. Overall, our findings suggest that BPA and 4-NP can induce stress responses and also modulate steroid hormone receptor-mediated signaling pathways. However, the specific response mechanisms of *Hsps* to EDs remain to be investigated in daphnids.

Heat map and hierarchical clustering analysis

To construct a heat map, an additional three antioxidant enzymes (*Cu/Zn-SOD*, *Mn-SOD*, and *CAT*) (Kim et al. 2017) were analyzed with eleven genes. Transcriptional profiles showed different patterns in BPA and 4-NP, indicating their different reaction mechanisms. A clustering analysis revealed no significant grouping in BPA exposure for 24 h (Fig. 3a), whereas several *GSTs*, *GRx*, and *GPx* were grouped with the smallest distance in BPA exposure for 48 h (Fig. 3b). GPx, GSTs, and GRx are included in a GSH-dependent system that requires GSH as a cofactor. In particular, GSH-dependent systems were strongly induced

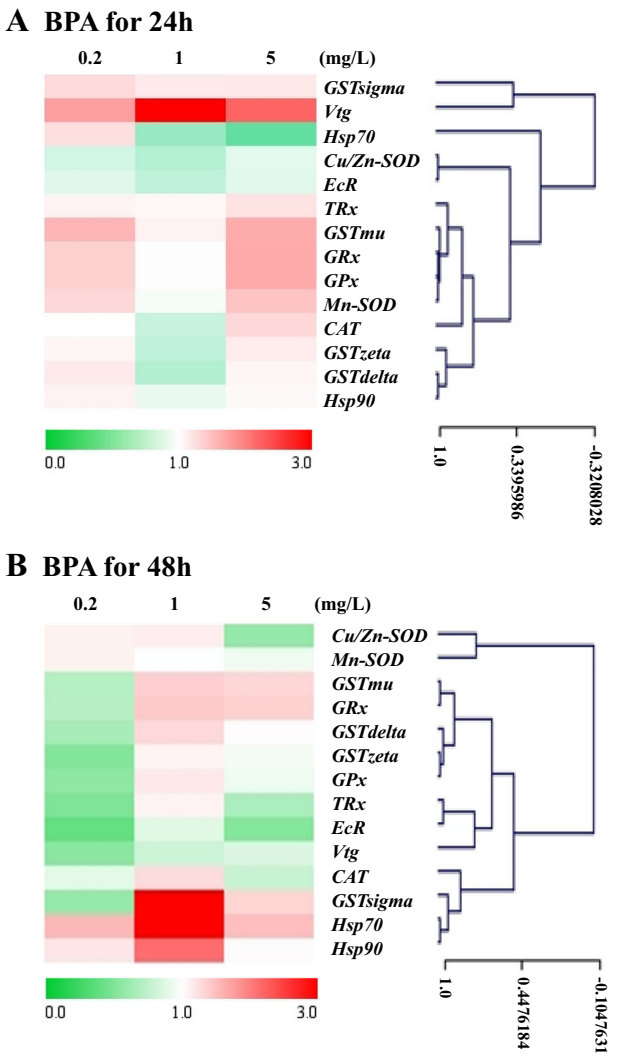

Fig. 3 Transcriptional profiles of eleven molecular biomarker genes with hierarchical cluster analysis in Daphnia magna - exposed to BPA (0.2, 1, and 5 mg/L) for 24 h and 48 h, respectively. A heat map was constructed to present the mRNA expression patterns using MeV software

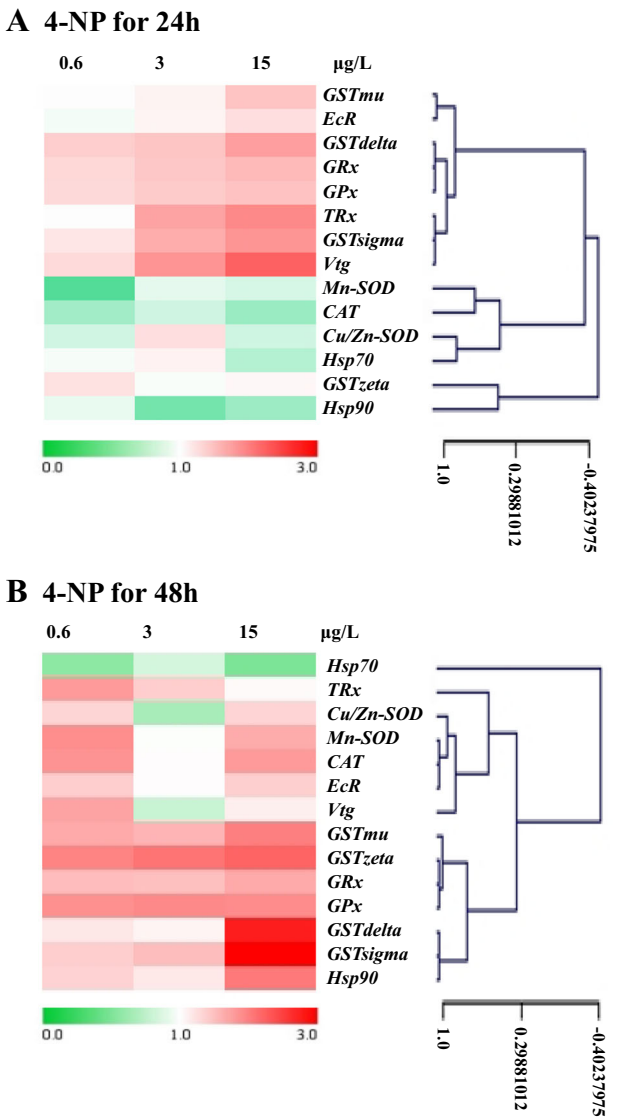

Fig. 4 Transcriptional profiles of eleven molecular biomarker genes with hierarchical cluster analysis in Daphnia magna - exposed to 4-NP (0.6, 3 and 15 mg/L) for 24 h and 48 h, respectively. A heat map was constructed to present the mRNA expression patterns using MeV software

in clustering by 4-NP exposure for 24 h and 48 h (Fig. 4a, b). Their activation in response to BPA and 4-NP may cause depletion of cellular GSH levels and result in oxidative stress, like *Bellamya purificata* (Li et al. 2008) and *Bombyx mori* (Yuan et al. 2013). In particular, Yuan et al. (2013) also demonstrated a relationship between 4-NP and GSH-related enzymes in *B. mori*. Taken together, these findings indicate that GSH-related genes may be potential molecular markers for evaluating EDs-mediated toxicity in daphnids.

Conclusions

In the present study, we report that BPA and NP can modulate transcription of genes related to the detoxification, antioxidant, development/reproduction, and stress with different modes of action in *D. magna*. Our findings provide a better understanding molecular mode of action of BPA and 4-NP in daphnids. In addition, our results suggest that multiple molecular biomarkers could be applied to evaluate ED-mediated toxicity using a model species. Further studies are required to unveil the molecular mechanism related to signaling pathways affected by BPA and 4-NP exposure in daphnids.

Acknowledgements This work was supported by a grant from the National Research Foundation of Korea (NRF-2016R1A2B4009939) funded to Young-Mi Lee.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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