



Effects of water accommodated fractions (WAFs) of crude oil in two congeneric copepods *Tigriopus* sp.



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ABSTRACT

Oil pollution has deleterious effects on marine ecosystems. However, the toxicity of crude oil towards Antarctic marine organisms has not been well studied. We compared the deleterious effects of water accommodated fractions (WAFs) of crude oil on reproduction, intracellular reactive oxygen species (ROS) levels, and antioxidant enzymatic activity in Antarctic (*Tigriopus kingsejongensis*) and temperate (*Tigriopus japonicus*) copepods. Reproductive rates of *T. kingsejongensis* and *T. japonicus* were significantly reduced ($P < 0.05$) in response to WAFs. Furthermore, *T. kingsejongensis* showed elevated levels of ROS and higher antioxidant enzyme (glutathione peroxidase [GPx]) activity than *T. japonicus* in response to WAFs. *CYP* genes from congeneric copepods were identified and annotated to better understand molecular detoxification mechanisms. We observed significant up-regulation ($P < 0.05$) of *Tk-CYP3024A3* and *Tj-CYP3024A2* in response to WAFs, suggesting that *CYP* genes may contribute to the detoxification mechanism in response to WAF exposure. These findings also suggest that WAFs may induce oxidative stress, leading to reproductive impairment in copepods. Furthermore, *Tk-CYP3024A3* and *Tj-CYP3024A2* genes can be considered as potential biomarkers of WAF toxicity in the congeneric copepods *T. kingsejongensis* and *T. japonicus*. This study will be helpful for enhancing our knowledge on the harmful effects of WAFs in Antarctic and temperate copepods and provides insight into the underlying molecular mechanisms.

1. Introduction

Antarctica is one of the most pristine and unpolluted marine ecosystems on earth. However, petroleum-related human interventions in recent years have increased concerns about the deleterious effects of oil pollution on Antarctic marine ecosystems (Aislabie et al., 2004; Oil Tanker Spill Statistics, 2009; Australian Maritime Safety Authority, 2012). Many accidental oil spills have occurred, and have resulted in severe damage to ecosystems (Cripps and Priddle, 1991; Ansaldo et al., 2005; Bargagli, 2008; Alexander et al., 2017).

Crude oil is a heterogeneous mixture and contains several compounds (i.e. polyaromatic hydrocarbons [PAHs], alkylated PAHs, and non-hydrocarbons) (Volkman et al., 1994; Salar Amoli et al., 2006). Of these, PAHs are considered to be carcinogenic, and therefore, that can cause serious damage to diverse marine organisms (Xue and Warshawsky, 2005). Furthermore, PAHs can lead the formation of reactive oxygen species (ROS) that induce oxidative stress (Parti et al., 2009). For example, ROS generation was observed in the liver tissue of the fish *Carassius auratus* in response to phenanthrene (Yin et al., 2007).

In the copepod *T. japonicus*, ROS was significantly increased in response to β -naphthoflavone (β NF) (Rhee et al., 2015). This stress has been identified as one of the reasons for its adverse effects on the normal physiologies (e.g. growth and reproduction) of marine organisms in laboratory studies (Peterson et al., 2003; Salar Amoli et al., 2006; Jernelöv, 2010). Water accommodated fractions (WAFs) of crude oil have been used in the laboratory setting to study the harmful effects of crude oil on model organisms. The acute toxicity of WAFs was evaluated in the key Arctic species; the copepod *Calanus glacialis*, juvenile Arctic cod (*Boreogadus saida*), and larval sculpin (*Myoxocephalus* sp.), and found that the relative sensitivity was similar without much discrepancies among the species (Gardiner et al., 2013). Furthermore, WAFs have been found to have adverse effects on survival, growth, and reproduction with alteration of cellular molecular responses in marine organisms like the marine medaka *Oryzias melastigma* (Mu et al., 2014), copepods *Calanus finmarchicus*, *C. glacialis* (Hansen et al., 2011), *Tigriopus japonicus* (Hwang et al., 2017), and *Paracyclops nana* (Puthumana et al., 2017a). These studies suggest that WAFs have effects at multiple biological and ecological levels from molecules to

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individuals. More detailed information on oil toxicity and associated physiological and molecular alterations in marine invertebrates have been published in recent years (reviewed by Han et al., 2017). However, no previous study has compared the toxic effects of WAFs between Antarctic and temperate marine invertebrates.

Among marine invertebrates, copepods function as a bridge between producers and consumers, allowing transfer of energy and toxicants. They are therefore considered promising model species for environmental genomic and marine ecotoxicology (Theilacker and Kimball, 1984). The copepod genus *Tigriopus* is distributed worldwide, and the *T. japonicus* (temperate species) has been widely used as an appropriate laboratory model species of the marine environment due to the small size (< 1 mm), short life cycle (< 2 weeks), and easy maintenance (Raisuddin et al., 2007). Recently, the Antarctic copepod *T. kingsejongensis* (Copepoda: Harpacticoida: Harpacticidae) was considered as a suitable model species for Antarctic ecophysiological and ecotoxicological research (Park et al., 2014; Kim et al., 2016a, 2016b; Lee et al., 2016). In our previous study, we found that the Antarctic and temperate copepods *T. kingsejongensis* and *T. japonicus* had different susceptibilities to UV radiation by examining the expression of genes such as glutathione S-transferase (*GST*) and heat shock proteins (*Hsps*) (Han et al., 2016). Comparative toxicity studies of these congeneric species will likely provide a better understanding of molecular and ecological responses to marine pollution in these two climatic regions.

Cytochrome P450 (*CYP*) enzyme families are large and diverse enzyme groups associated with Phase I detoxification mechanisms in living organisms. They can recognize a wide variety of endobiotics and xenobiotics (e.g. drugs, chemicals, and hormones) as substrates (Snyder et al., 1998; Chaty et al., 2004). Furthermore, in the previous studies, *CYP* genes were used as biomarkers for environmental monitoring to detect oil spill and organochlorine pollution, as they were associated with detoxification mechanisms in marine invertebrates such as the copepods *T. japonicus* (Han et al., 2014a), *C. glacialis*, *C. hyperboreus* (Hoekstra et al., 2002) and *P. nana* (Han et al., 2015; Dahms et al., 2016) and the rotifer *B. koreanus* (Won et al., 2016). Thus, understanding the molecular response of *CYP* genes in Antarctic and temperate marine invertebrates to oil pollution is important to uncover the underlying detoxification mechanism and to determine if *CYP*s are suitable biomarkers for oil pollution in the polar region.

In this study, to determine if there was any difference in the toxic effects of WAFs on Antarctic versus temperate copepods, we investigated the reproduction rate and measured the cellular ROS level and enzymatic activity of GPx in *T. kingsejongensis* and *T. japonicus*. Furthermore, to evaluate the feasibility of using *CYP* genes as biomarkers to detect oil pollution in the Antarctic region, we identified 30 *CYP* genes from *T. kingsejongensis* and analyzed transcription of *Tk-CYP3024A3*, which was significantly elicited ($P < 0.05$) by WAFs (Han et al., 2014a). This comparative study will increase our understanding of the mechanistic effects of crude oil in Antarctic copepods.

2. Materials and methods

2.1. Culture and maintenance of *Tigriopus kingsejongensis* and *T. japonicus*

The copepods *T. kingsejongensis* (kindly provided by Dr. Sanghee Kim, Korea Polar Research Institute, Incheon, South Korea) and *T. japonicus* (collected from Haeundae beach (Busan, South Korea)) were maintained at 8 and 25 °C for *T. kingsejongensis* and *T. japonicus*, respectively (Han et al., 2016). The detailed explanation of the culture condition and maintenance of *Tigriopus kingsejongensis* and *T. japonicus* are incorporated in the Supporting Information as described in our previous studies (Han et al., 2016). Species identity was confirmed by morphometric analysis followed by molecular characterization of the universal marker cytochrome oxidase 1 (*COI*) gene (Park et al., 2014).

2.2. Comparative effect of WAFs on reproduction

WAFs of Iranian crude oil were prepared in accordance with Aurand and Coelho (2005). To investigate the response of WAF exposure on the rate of reproduction in *T. kingsejongensis* and *T. japonicus*, one ovigerous female was transferred to 4 ml solution (0 [control], 20%, 40%, and 80% WAF), respectively in a 12-well cell culture test plate (SPL Life Sciences, Seoul, South Korea) and were maintained at 8 and 25 °C for *T. kingsejongensis* and *T. japonicus*, respectively. This procedure was performed for 10 replicates. Every 24 h, the number of newly generated nauplii was calculated using a stereomicroscope (SZX-ILLK200, Olympus, Tokyo, Japan) for 10 days. During the experiment time, 50% of the test solution was renewed and 10 µl of *Tetraselmis suecica* (~ 6×10^4 cells/ml) once per day were given as live feed.

2.3. Measurement of ROS and antioxidant enzyme activity

We measured ROS levels and glutathione peroxidase [GPx] activity in *T. kingsejongensis* and *T. japonicus* exposed to various concentrations of WAFs (0 [control], 20%, 40%, 60%, and 80%) for 24 h to assess WAF-induced oxidative stress. For the quantitative measurement, total protein was measured by the Bradford method (Bradford, 1976). The ROS was assessed by following our previous paper (Kim et al., 2011) and the detailed protocol is given as Supporting Information. Enzymatic activity of GPx was calculated by using a GPx cellular activity assay kit (Sigma–Aldrich Co, St. Louis, MO, USA) and the reduction in absorbance relative to the control was measured using a spectrophotometer (Ultrospec 2100 Pro, Amersham Biosciences, Cambridge, England).

2.4. Annotation and phylogenetic analysis of *CYP* genes

We searched RNA-Seq library of *T. kingsejongensis* (Kim et al., 2016a, 2016b) to obtain *CYP* gene sequences and the annotated genes were deposited in GenBank. To determine the phylogenetic position, we used amino acid sequences of *CYP*s from three copepods – *T. kingsejongensis*, *T. japonicus*, and *P. nana* – using ClustalW followed by phylogenetic analysis using MrBayes (WAG-model, version 3.1.2) and viewed by TreeView version 1.6.6 module of PHYLIP (Han et al., 2015; for detailed protocol see Supporting information).

2.5. Acute toxicity tests in response to WAFs

Acute toxicity test (96-h) was performed using 10 adult copepods and exposed to test solutions (4 ml in a dish) with various concentrations of WAFs (0 [control], 40%, 60%, 80%, and 100%) prepared in ASW in triplicate under confined laboratory conditions used for acclimation. Copepods were not fed during the experiments. Test solutions were renewed once every 48 h. Mortality was recorded once every 24 h. From the percentage mortality and WAFs concentration, LC10-96h and LC50-96h values were calculated using Probit analysis (ToxRat® Ver.2.09, ToxRat Solutions GmbH, Alsdorf, Germany).

2.6. Effect of WAFs on transcription of *CYP* genes

We exposed ~ 30 copepods to various concentrations of WAFs (0 [control], 40%, 60%, and 80%) for different periods of time (0, 6, 12, 24, and 48 h) to assess the mRNA transcription level of *Tk-CYP3024A3* gene. Extraction of total RNAs and quantitative real-time RT-PCR (qRT-PCR) were performed in accordance with our previous studies (Han et al., 2014a) and according to the manufacturer's instructions. Detailed protocols were appended as Supporting information. All qRT-PCR experiments were performed in triplicate and the primers used are given in Table 1. Relative expression patterns of *Tk-CYP3024A3* gene mRNA in terms of fold difference was evaluated using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

Table 1

GenBank accession number and primer sets used for this study.

Gene (GenBank nos.)	Oligo name	Sequence (5' -> 3')
<i>Tk-3024A3</i>	F	CCATTTGCCCTTCACTCTTCG
(KY249914)	R	GGGAAATATTGGGGTCATAG
<i>Tj-3024A2</i>	F	GGACATTCTTACCCCTTGGC
(KF639994)	R	TCCATTTTATTGGAATCACAGTC
<i>Tk-18rRNA</i>	F	CACCGAACCACTGGCAATG
(KX695154)	R	AAAAGTCAGCTCGCACGGAC
<i>Tj-18rRNA</i>	F	TCGGGCTGTCTCGTTGGTGATTC
(EU054307)	R	TGCCACAGTCGACAGTTGATAGG

2.7. Statistical analysis

Data were analyzed using one-way ANOVA, followed by Tukey's posthoc analysis ($P < 0.05$). Levene's test was used for assessing the normal distributions and homogeneity of variances between samples. Data were mean values with standard errors (mean \pm SE). Analyses were done with SPSS® software (ver 21; SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effect of WAFs on reproduction of *T. kingsejongensis* and *T. japonicus*

We compared the reproductive rate of the congeneric copepods *T. kingsejongensis* and *T. japonicus* in response to WAF exposure for 10 days. The average number of *T. kingsejongensis* hatched nauplii was higher (~60) than that of *T. japonicus* (~30) in the control case. However, the total number of nauplii that hatched over 10 days was significantly higher ($P < 0.05$) in *T. japonicus* than *T. kingsejongensis* (Fig. 1) due to the short reproductive cycle of *T. japonicus*. In both cases, the reproductive rate decreased significantly ($P < 0.05$) in a concentration-dependent manner in response to WAF exposure. The WAFs composition is appended as Table S1.

3.2. Oxidative status in response to WAFs

To evaluate whether WAFs induced oxidative stress in *T. kingsejongensis* and *T. japonicus*, we analyzed the *in vivo* ROS level and GPx activity in response to WAF exposure. ROS level increased ($P < 0.05$) in *T. kingsejongensis* exposed to 20% WAFs, but in *T. japonicus*, the ROS level increased ($P < 0.05$) only at 80% WAF (Fig. 2A). Similarly, the antioxidant enzyme GPx was found to be induced in *T. kingsejongensis*, whereas no increased GPx enzyme activity was observed in *T. japonicus* (Fig. 2B).

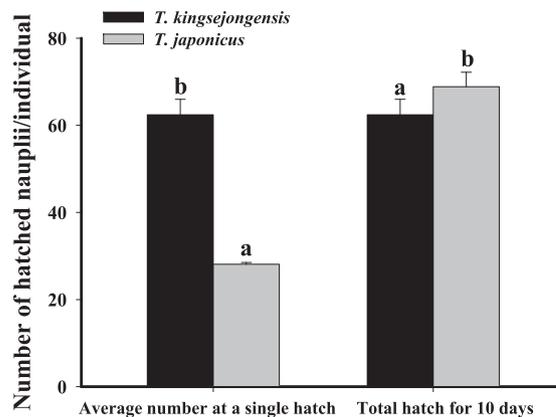
3.3. Acute toxicity test

To calculate LC50-96h and NOEC values, we measured the endpoint of survival and death at different concentrations of WAFs (0, 20, 40%, 60%, 80%, and 100%) for 96 h. No mortality was observed for any WAF concentration including 100% WAF (unpublished data).

3.4. Annotation and phylogenetic analysis

We annotated 30 CYP genes from the RNA-seq databases of *T. kingsejongensis* according to Dr. David R. Nelson (Cytochrome P450 Nomenclature Committee) and submitted to GenBank (Table S2). We had previously annotated 52 CYPs from *T. japonicus* and included these genes in the phylogenetic analysis along with 46 CYPs from *P. nana*. *Tk*-CYPs and *Tj*-CYPs were found to be clustered with a group of five CYP clans (clan 2, 3, 20 and mitochondrial clan [MT]), and CYPs from the copepod *P. nana* (Fig. S2). Interestingly, we found that the CYP20A1 grouped in a separate clan, clan 20. Review of the multiple sequence alignment confirmed the presence of conserved heme-binding signature

A) Average hatched nauplii/individual



B) Average daily hatched nauplii for 10 days

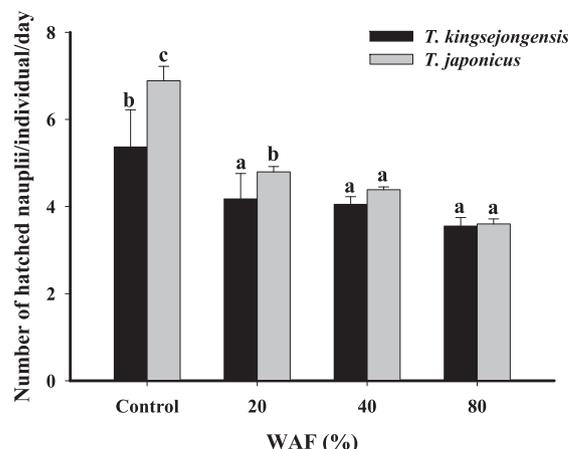


Fig. 1. The reproduction rate of the copepod *Tigriopus kingsejongensis* compared with that of *Tigriopus japonicus*. (A) Average hatched nauplii at one time and total numbers of hatched nauplii of ovigerous females over 10 days, (B) Effect of WAFs on daily nauplii production of ovigerous females over 10 days on average. Significant differences were analyzed by ANOVA (Tukey's post hoc test $P < 0.05$). Data are presented as the mean \pm SE. The letters (a, b, and c) indicate significant differences, respectively.

domains in the CYPs (Fig. S3).

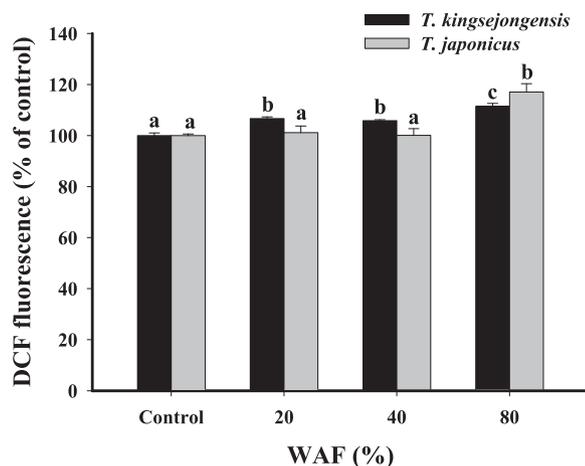
3.5. Transcription of CYP genes in response to WAFs

We measured the mRNA expression of *Tk-CYP3024A3* and *Tj-CYP3024A2* (clan 3; CYP3024 subfamily) to compare the effects of WAFs. Expression of *Tk-CYP3024A3* and *Tj-CYP3024A2* was elicited significantly ($P < 0.05$) in *T. kingsejongensis* and *T. japonicus* in response to WAFs, respectively (Fig. 3). However, the expression of *Tj-CYP3024A2* was concentration-independent and was significantly evoked ($P < 0.05$) at 80% WAF from 6 to 48 h with maximum mRNA expression level at 6 h, whereas *Tk-CYP3024A3* was significantly induced ($P < 0.05$) at 80% WAF from 12 h with the highest elevated response at 24 h.

4. Discussion

Accidental oil spills are causing widespread and long-lasting damage to marine ecosystems around the world (Fukuyama et al., 2000; Pérez-Cadahía et al., 2004; Jernelöv, 2010). However, similar incidents are relatively less common in the polar areas, and thus little

A) ROS (for 24 h)



B) GPx (for 24 h)

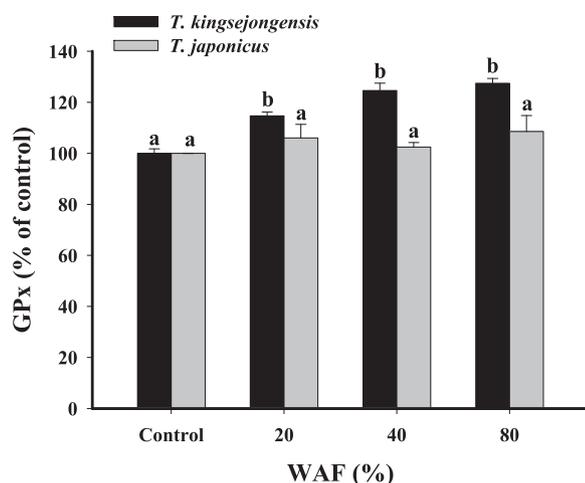


Fig. 2. Generation of (A) ROS and measurement of (B) GPx enzymatic activity in response to WAF exposure (0 [control], 20, 40, 80%) for 24 h. The significance of differences was assessed by ANOVA (Tukey's post hoc test, $P < 0.05$). The letters (a, b, and c) indicate significant differences, respectively.

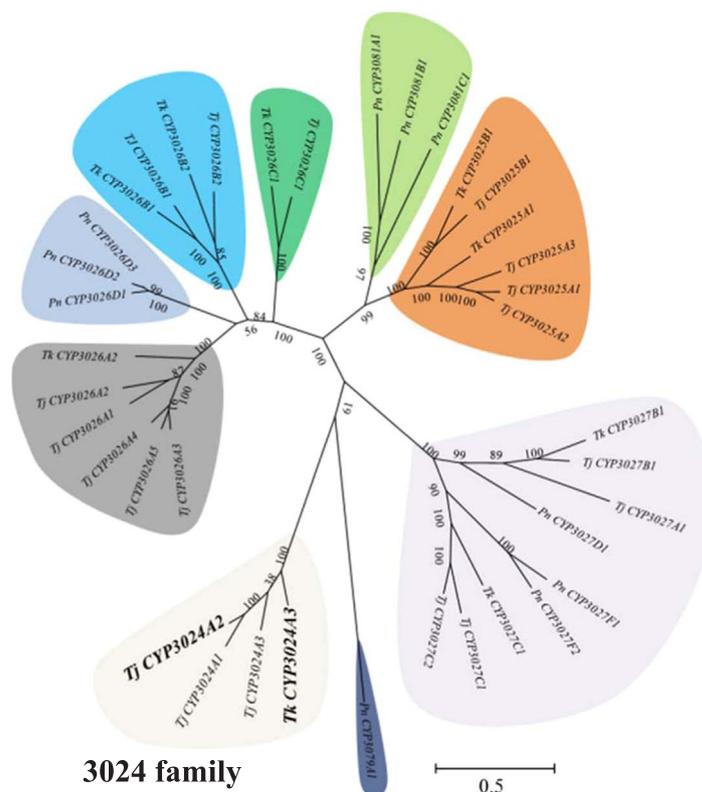
information is available concerning the toxicity of oil towards polar marine organisms (Hansen et al., 2011; Gardiner et al., 2013). Through this study, we compared the harmful effects of oil pollution on the two congeneric copepods species collected from the Antarctic and a temperate region, respectively (Fig. S1). In general, physical factors (e.g. temperature and salinity) are well known key external factors affecting the biological processes of organism at the individual, population, community, and the ecosystem levels (Pörtner and Knust, 2007). For example, adverse effects on reproductive performance were observed in increasing temperature in the hermaphrodite fish *Kryptolebias marmoratus* (Park et al., 2017). Also, retardation of development and fecundity was demonstrated in response to salinity changes in the copepod *P. nana* (Lee et al., 2017; Puthumana et al., 2017b). Antarctic and temperate copepods had different reproductive rates (Fig. 1A). In particular, the average number of hatched nauplii of *T. kingsejongensis* was lower than that of *T. japonicus* after a period of 10 days, although the average number of hatched nauplii was greater per *T. kingsejongensis* individual than per *T. japonicus* individual. Regardless of physical factors, the reproductive rates of *T. kingsejongensis* and *T. japonicus* were significantly reduced ($P < 0.05$) in response to WAF (Fig. 1B). Previous studies reported that WAFs resulted in reproductive impairment of

diverse marine organisms. For example, in the estuarine amphipod *Corophium volutator*, reduced reproduction was observed in response to WAFs (Scarlett et al., 2007). Also, reduced fertilization success associated with a WAF-associated reduction in sperm concentration was observed in the polychaetes *Nereis virens* and *Arenicola marina* (Lewis et al., 2008), implying that WAFs can have adverse effects on the reproductive system of *T. kingsejongensis* and *T. japonicus*, and could possibly lead to population reduction and changes in Antarctic and temperate marine ecosystem dynamics (Edwards and Richardson, 2004). In this study, we also found that intracellular ROS level and GPx enzymatic activity, which are associated with oxidative stress and antioxidant defense mechanisms (Pastore et al., 2003; Birben et al., 2012), were increased in the WAF-exposed copepods, although no significant changes in GPx enzymatic activity was found in *T. japonicus* regardless of WAF concentration (Fig. 2). ROS levels in *T. kingsejongensis* were increased at all WAF concentrations (0, 20, 40, 80% WAF), while the ROS level in *T. japonicus* was only increased by exposure to a high WAF concentration (80%), indicating that *T. kingsejongensis* is more sensitive than *T. japonicus* to WAFs. We previously reported that the two congeneric copepods *T. kingsejongensis* and *T. japonicus* had different susceptibilities to UV radiation with regard to survival rate, ROS level, and antioxidant enzymatic activity (Han et al., 2016), indicating that these two congeneric copepods have a species-specific response and tolerance associated with oxidative stress induced by environmental stressors. In general, ROS are well-known inducers of oxidative damage, as it can damage important macromolecules (DNA, proteins, and lipids) leading to adverse effects (e.g. cellular damage, mutagenesis, and carcinogenesis) (Valko et al., 2007). To date, ROS-mediated antioxidant enzymes (e.g. GST, GPx and GR) have been considered as biomarkers of environmental stressors (e.g. gamma radiation and WAF) to confirm the effects on developmental, survival, and reproductive parameters in marine organisms. Previous studies reported that elevated ROS and antioxidant enzymes were associated with growth retardation and reproductive impairment in the gamma irradiated copepods *P. nana* (Won and Lee, 2014) and *T. japonicus* (Han et al., 2014b). In the rotifer *B. koreanus*, an increased level of ROS, which was associated with reduced lifespan and fecundity, was found in response to WAF exposure (Won et al., 2016), suggesting that WAFs can induce oxidative stress with detrimental effects on fecundity.

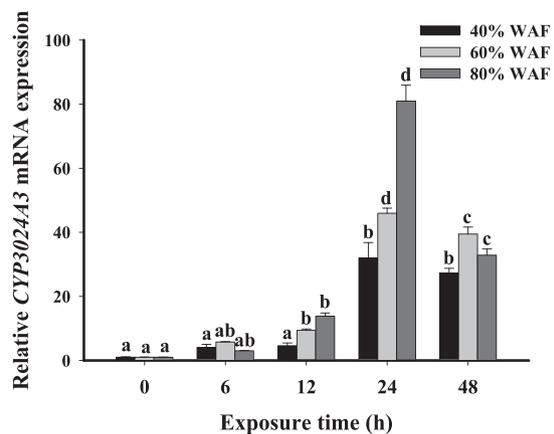
Mortality was not observed at any WAF concentration in two congeneric copepods (unpublished data), although impaired reproduction was observed. WAFs are composed mainly of highly soluble and bioavailable low molecular weight PAHs and alkylated PAHs that have toxic effects on marine organisms (Basu et al., 2001). Alkylated PAHs and 16 PAHs are the major components of WAFs. Moreover, the ratio of phenanthrene, fluorene, and alkylated naphthalene are rich in WAFs of Iranian crude oil (Han et al., 2015; Hwang et al., 2017; Table S1). Irrespective of their precise nature, WAFs have been shown to have different toxic effects in various copepod species (reviewed from Han et al., 2017). Alkylated PAHs induce more toxic effects in copepods than the parental PAH (Han et al., 2015). Furthermore, the major chemicals in WAFs are highly lipophilic and therefore highly reactive towards cellular components. Thus, WAFs compositions are toxic to copepods, irrespective of the crude oil in which the WAFs are originated.

Previous studies suggested the use of cellular energy allocation (CEA) as an indicator of the harmful effects of oxidative status on reproduction and self-maintenance in the water flea *Daphnia magna* (Calow, 1991; De Coen and Janssen, 2003). Also, environmental stress can trigger energy re-allocation towards antioxidant defense and repair mechanisms (De Coen and Janssen, 1997; Novais et al., 2013; Gomes et al., 2015). Indeed, exposure of *P. nana* (Han et al., 2015) and *B. koreanus* (Won et al., 2016) to WAFs, even though it resulted in detrimental effects on life cycle parameters (e.g. growth and reproduction), did not affect mortality (even 100% WAF), which suggests that these organisms may have a survival strategy that involves energy re-

A) Phylogeny of copepod CYP genes (Clan 3)



B) *Tk-CYP3024A3*



C) *Tj-CYP3024A2*

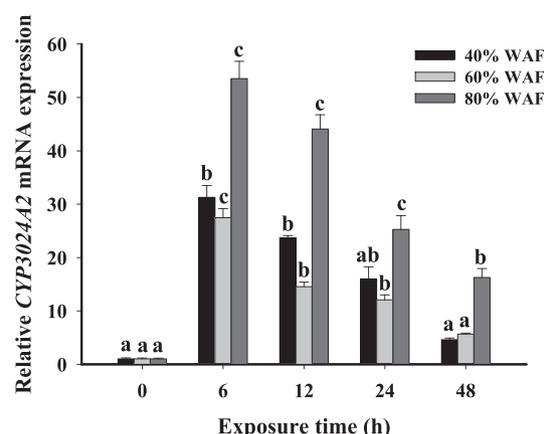


Fig. 3. (A) Phylogenetic placement of select *Tk-CYP* genes with WAF-sensitive *CYP* genes of *T. japonicus*. The mRNA expression of *Tk-CYP3024A3* (B) and *Tj-CYP3024A2* (C) in response to different concentrations of WAFs (control, 40%, 60%, and 80%) are shown as relative to *18S rRNA*. The significance of differences was analyzed by ANOVA (Tukey's post hoc test, $P < 0.05$).

allocation to adaptation and/or homeostatic maintenance from normal physiological processes in response to oxidative stress generated by WAFs.

We identified 30 *CYP* genes with full-length cDNAs from the Antarctic copepod *T. kingsejongensis* and compared with *CYP* genes (52) of the congeneric species, *T. japonicus*. Moreover, we analyzed variations in the mRNA transcription levels of *CYP* genes induced by WAFs, to confirm whether *CYP* genes can be used as biomarkers to assess oil pollution in Antarctic and temperate marine ecosystems. First, we

found that the 30 *Tk-CYP* and 52 *Tj-CYP* genes were diverted to four clans (clan 2, 3, 20, and clan MT) (Fig. S2). The phylogenetic tree of *CYP* genes was similar to that of *P. nana* (Han et al., 2015). *CYP20A1* genes (clan 20) were also found in the two congeneric copepods. To date, *Tj-CYP20A1* has an undefined biological function as well as substrate specificity, thus, it is also known as an “orphan P450” (Guengerich et al., 2005). Previously, high expression of *CYP20A1* was observed in the vertebrate brain including human, suggesting that it is likely to be involved in neurophysiological functions (Stark and

Guengerich, 2007; Stark et al., 2008; Goldstone et al., 2010). Furthermore, *CYP20A1* mRNA transcripts are associated with embryonic development in the zebrafish (Goldstone et al., 2010), although *CYP20A1* from invertebrates has not yet been functionally characterized. However, further studies at the molecular level are needed to explicate function of *CYP20A1* in arthropods.

All the 30 *Tk-CYP* genes had heme-binding motif FXXGXXXXCXG (Fig. S3) that is necessary for oxidative hydroxylation reactions (Werck-Reichhart and Feyereisen, 2000). The heme-binding motif is conserved among bacterial, plant, and mammalian *CYP* genes (Nelson, 1999; Chapple, 1998; Otyepka et al., 2007). In the copepod *P. nana*, 46 *CYP* genes with a similar heme-binding domain were identified (Han et al., 2015). This heme-binding motif has also been reported from two *Salvelinus* species (lake trout *S. namaycush* and brook trout *S. fontinalis*) and the polychaete *Perinereis nuntia*, despite dissimilarities in the gene structure (Rees and Li, 2004; Won et al., 2013). The conservation of this motif reflects the important role of *CYP* gene products in transferring protons during monooxygenation.

In this study, we found that expression of *Tk-CYP3024A3* was found evoked by WAFs compared with that of *Tj-CYP3024A2*, which is in the same *CYP3024* subfamily (Fig. 3). *CYP1* gene members have been used in vertebrates as biomarkers of environmental pollutants because of their role in the aryl hydrocarbon receptor (AHR)-*CYP* pathway (Stegeman et al., 1986; Fujita et al., 2001; Lambert et al., 2006). However, the *CYP1* gene family does not exist in invertebrates, suggesting that they have a diverse mechanism of xenobiotic-mediated *CYP* activation (Kim et al., 2015) and thus, different biomarkers of toxicity are essential. Expression of *CYP431A1*, *CYP432A1*, and *CYP4DU1* was significantly increased in the WAF-exposed polychaete *P. nuntia* (Won et al., 2013). In the rotifer *B. koreanus*, expression of *CYP3045C1* mRNA was highly up-regulated in response to WAFs (Won et al., 2016). In addition, the *CYP3027F1* and *CYP3027F2* genes of the copepod *P. nana* were up-regulated by WAFs induction similar to the three *CYPs* identified from *T. japonicus* (*Tj-CYP3024A2*, *Tj-CYP3024A3*, and *CYP3024C2*) belong to the same clade (Han et al., 2014a, 2015). In addition, AhR and aryl hydrocarbon receptor nuclear translocator (ARNT) genes were identified and showed significant change ($P < 0.05$) of their gene expression patterns in polychlorinated biphenyl (PCB)- and benzo[α]pyrene (B[α]P)-exposed *T. japonicus* (Kim et al., 2015) and WAF-exposed to *P. nana* (Han et al., 2015). Furthermore, response elements for several transcription factors (e.g., aryl hydrocarbon-responsive elements [AhREs]; 5'-GCGTG-3') were found in the regulatory regions of the three *CYP* genes in *T. japonicus* (Han et al., 2014a), suggesting that *T. kingsejongensis* *CYPs* associated with AHR-*CYP* pathway (Fig. S4) may be involved in detoxification of xenobiotics as shown for the copepods *T. japonicus* and *P. nana*. Taken together, *Tk-CYP3024A3* and *Tj-CYP3024A2* are potential biomarkers with which to evaluate oil pollution in Antarctic and temperate regions.

In summary, we have envisaged to identify the biomarkers from congeneric copepods to detect early-warning signs in the crude oil-exposed copepods. Also, we have focused on developing *T. kingsejongensis* as a model copepod to study the immediate effects of oil pollution at Antarctic region. Thus, we exposed high concentration of WAFs with sub-lethal effects on copepods for a shorter period of exposure (48 h). Overall, WAFs had adverse effects on reproduction in two congeneric copepods, *T. kingsejongensis* and *T. japonicus*, and this was likely mediated by an increase in oxidative stress. *Tk-CYP3024A3* was increased significantly by WAFs induction for a shorter period of exposure (48 h) in addition to *Tj-CYP3024A2*, indicating that these *CYP* genes are linked with the detoxification mechanisms and are potential biomarkers of oil pollution in Antarctic and temperate regions. These findings will advance our knowledge on the harmful effects of WAFs in Antarctic and temperate copepods and provide insight into the underlying molecular mechanisms.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ecoenv.2017.07.065>.

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