Agr. Nat. Resour. 53 (2019) 652–661

AGRICULTURE AND NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

18S rRNA, a potential reference gene in the qRT-PCR measurement of bisphenol A contamination in green mussels (*Perna viridis*) collected from the Gulf of Thailand

Chuta Boonphakdee[a,b,c,*], Yhardpeth Ocharoen[a,c], Andrew P. Shinn[d], Santi Suanla[b], Jeeranan Thamnawasolos[b]

[a] *Graduate Program in Environmental Science, Faculty of Science, Burapha University, Chonburi 20131, Thailand*

[b] *Department of Biology, Faculty of Science, Burapha University, Chonburi 20131, Thailand*

[c] *Center of Excellence on Environmental Health and Toxicology (EHT), Faculty of Science, Mahidol University, Bangkok 10400, Thailand*

[d] *Fish Vet Group Asia Limited, Saensook, Chonburi 20130, Thailand*

Article Info

Article history:
Received 22 June 2018
Revised 7 August 2018
Accepted 15 August 2018
Available online 25 December 2019

Keywords:
Endocrine disruptor,
Environmental pollution,
Gene expression,
Marine bivalves,
Normalization

Abstract

Normalization of reference genes through the selection of appropriate housekeeping genes is important for the reliable quantification of mRNA analyzed using real-time polymerase chain reaction (qRT-PCR). The suitability of various housekeeping genes (HKGs), were valuated as internal references to verify the levels of the contaminant bisphenol A (BPA, 2,2-bis (4-hydroxyphenyl) propane) by determining the level of gene expression of *cytochrome P450 4* (*cyp4*) within the digestive gland of green mussels (*Perna viridis*)—a species used for biomonitoring environmental levels. Additionally, the effect was explored of mussel gender and maturity status (juvenile or mature) on the stability of the expression of the reference genes. Three candidate HKGs were examined, namely *18S rRNA, 28S rRNA* and *β-actin*. The expression stability was calculated using three statistical approaches—NormFinder, BestKeeper and a comparative threshold cycle (Ct) method. The study found that of the three candidate reference genes, *28S rRNA* and *18S rRNA* were the most stable with no significant differences when maturity, gender and exposure to BPA were considered. It was noted that when these genes (*18S rRNA*, *28S rRNA*, *β-actin*) were used for normalization using animals exposed to 10 ng BPA and the qRT-PCR method, different levels of *cyp4* expression were observed. However, BPA significantly decreased the expression levels of *cyp4* compared to the control (animals not exposed to BPA), only when *18S rRNA* was used as an internal reference. These results highlighted the importance of selecting appropriate reference genes as the endogenous control in qRT-PCR in biomonitoring programs of BPA.

Introduction

The recent increase in industrial activity in the eastern region of Thailand, especially along the coastal provinces of the Gulf of Thailand, has resulted in a marked increase in the amount of polycarbonate-based plastic waste produced by human activity. For example, the Map Ta Phut Industrial Estate situated in Rayong province, where many polycarbonate plants are located, is the largest hub of producers in Thailand. Among these, one of the main manufacturers (name withheld) produces approximately 290,000 t of polycarbonate-based plastic each year. The primary monomer raw material involved in the production is bisphenol A (BPA) or 2,2-bis

* Corresponding author.
E-mail address: chuta@buu.ac.th (C. Boonphakdee).

https://doi.org/10.34044/j.anres.2019.53.6.13

(4-hydroxyphenyl) propane. BPA is an organic compound used in the production of polycarbonates and epoxy resin that are two major components in plastic applications such as in the inner lining of water containers (Salgueiro-González et al., 2012). BPA has been known to leak into the environment from industrial and municipal sites and when present in high concentrations, it can cause adverse effects on marine organisms (Oehlmann et al., 2006; Flint et al., 2012; Canesi and Fabbri, 2015; Mathieu-Denoncourt et al., 2015; Xue and Kannan, 2016) and aquatic ecosystems. BPA is a xenoestrogen with properties able to mimic natural estrogen; its impacts on human health have been studied extensively but opinion is still very much divided as to the levels that exert adverse effects (Chouhan et al., 2014). Thus, in evaluating the effects of BPA contamination on various aquatic organisms, biomarkers have proved to be very useful (Schettino et al., 2012). The molecular processes occurring within an organism following pollutant exposure can provide a highly sensitive, early warning system of toxicological effects within individual populations. Green mussels (*Perna viridis*), which are cultured extensively throughout the Gulf of Thailand and Southeast Asia, as a sentinel organism that can bioaccumulate BPA, serve as an appropriate species to assess the potential effects of various toxicants (Ruangwises and Ruangwises, 1998; Boonyatumanond et al., 2002; Monirith et al., 2003; Fung et al., 2004; Isobe et al., 2007).

In eco-toxicological studies, quantitative real-time polymerase chain reaction (qRT-PCR) is considered a highly sensitive tool for the quantification of mRNA transcripts of select genes used as biomarkers in species affected by environmental contamination (Bustin, 2002; Dermitzakis, 2008; Maier et al., 2009). To obtain accurate and reliable mRNA transcript results of the sensitive qPCR method, proper normalization of internal reference genes is essential. Typically, one or more housekeeping genes are selected to be referenced against a target gene of interest because they normally display minute variations in expression levels across various different development phases, tissue types and environmental conditions (Wong and Medrano, 2005). Among these, the ribosomal RNA genes (*18S rRNA* and *28S rRNA*) and a cytoskeleton actin gene (*β-actin*) are commonly used as endogenous references in the qRT-PCR method. However, the selection of unsuitable reference genes for normalization can increase the error in the analysis of the test results (Wong and Medrano, 2005). Hence, accurate screening for suitable reference genes can result in minimizing the overall analytical costs.

Therefore, the aim of the current study was to determine the utility of three potential reference genes (*18S rRNA, 28S rRNA*, *β-actin*) for qRT-PCR using green mussels as a bio-indicator of bisphenol A contamination. Additionally, the influence was investigated of the different sexes and developmental stages (juvenile and mature) of these marine bivalves on the expression stability. The relative expression was quantified of the *cytochrome P450 4 (cyp4*) gene encoding the cytochrome P450 (CYP4) enzyme participating in the xenobiotic transformation by normalization with the candidate reference genes. Therefore, the aim of the study was to identify an appropriate reference gene for qRT-PCR use in programs applied for biomonitoring the levels of BPA.

Materials and Methods

Sample collection and maintenance

Juvenile (2 ± 0.4 cm shell length; n = 60) and mature (5 ± 0.5 cm shell length, approximately 1 g dry shucked weight; n = 90) green mussels were obtained from a mussel farm (details not disclosed) in Trat province, Thailand in January 2017 and transported within 6 hr to the hatchery of the Department of Aquatic Science, Faculty of Science, Burapha University. This site had served as the reference site in a previous study (Ocharoen et al., 2018) where the levels of BPA contamination within the Gulf of Thailand were found to be low (1.21 ± 0.01 ng/L BPA in seawater). The mussels were then conditioned in a 160 L glass tank with artificial seawater (30 parts per trillion, 120 L) and gently aerated using four evenly spaced 3.8 cm^2 air stones. The animals were fed twice daily (0900 hours and 1500 hours) with the diatom *Chaetoceros* sp. for 1 wk before starting the experiment. The number of mussels subsequently used for study is summarized in Fig. 1.

Experiment 1: Assessment of reference genes and maturity status of green mussels

The shells of 50 mature and 50 juvenile mussels were opened and the mussels were sexed. Three males and three females of each maturity status were then combined as one pool (n = 6). Triplicate samples of six mussels were prepared for each maturity stage (Fig. 1).

Experiment 2: Assessment of reference genes and mussel gender

From the mussels that were sexed for the first experiment, three triplicate pools each containing six males were processed alongside three triplicate pools of six females (Fig. 1).

Experiment 3: Assessment of reference genes on exposure to bisphenol A

The remaining 40 mature mussels were divided into four pools of 10 mussels, with two pools serving as the treatment groups and the remaining two pools as their corresponding controls (Fig. 1). The control group was given a 100 µL injection of 0.03% dimethyl sulfoxide (DMSO) in artificial seawater, while the treatment group was injected with 100 µL containing 0.03% DMSO+10 ng BPA in artificial seawater. A 1 mL sterile 27Gx½" U-100 insulin syringe (NIPRO; Thailand) was used to inject each individual mussel in the adductor muscle. One control pool and one treatment pool of mussels were then dissected to give a time zero sample (T_0). The digestive gland from each mussel was removed and sub-sampled to produce three pseudo-replicated samples each consisting of material excised from six mussels. The samples were then submitted for RNA extraction and qRT-PCR analysis. The remaining two groups of mussels were stocked into separate glass aquaria (20 cm × 32 cm × 20 cm) and maintained for 12 hr before they were processed in the same manner as the T_0 sample.

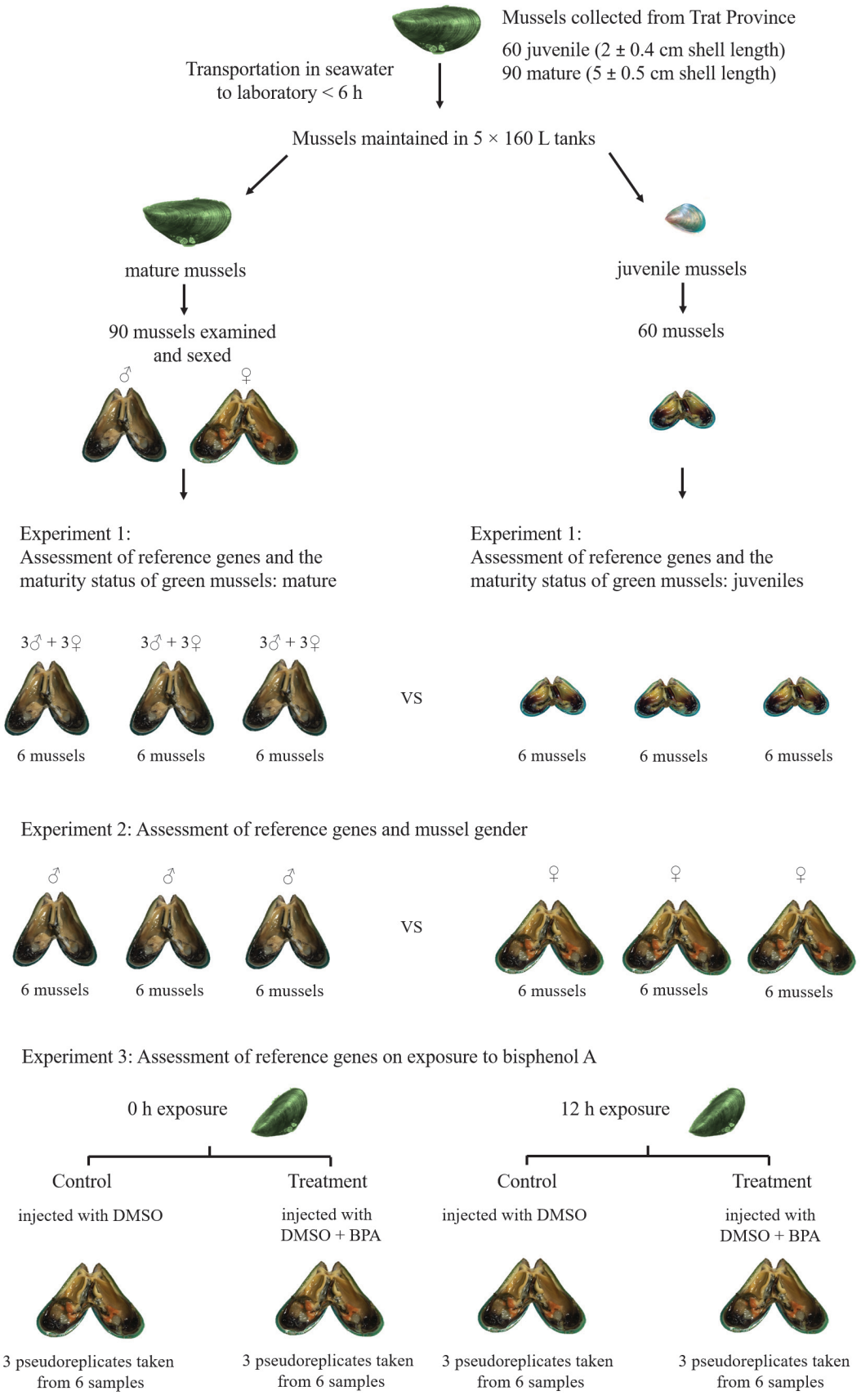

Fig. 1 Summary of experimental design used, where DMSO = dimethyl sulfoxide, BPA = 2,2-bis (4-hydroxyphenyl) propane

Total RNA extraction and cDNA synthesis

The total RNA was extracted from the digestive glands of each individual mussel (0.2 g/sample) using TRIzol reagent (Invitrogen; USA) and then further treated with RNase-free DNase I (Thermo Fisher Scientific; USA) to remove any genomic DNA contamination, following the manufacturer's protocols. The quantity and quality of the RNA were then determined using Nanodrop® 2000 (Thermo Fisher Scientific; USA) at absorbances of 260 nm and 280 nm. The samples with A260/280 values ≥ 2.0 were then selected for further analysis. One microgram of RNA from each sample was then converted into complementary DNA (cDNA) using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific; USA) as per the kit instructions. The cDNA was typically diluted 10-fold (except for the real-time PCR amplification efficiency test where 1 µL was used directly) with nuclease-free distilled water; 1 µL was then submitted for qRT-PCR analysis in a final volume of 15 µL.

Selection of candidate reference genes and primer design

A comparison was conducted between three common housekeeping genes—(cytoskeletal *β-actin (β-actin*) and two ribosomal RNA genes (*18S* and *28S*). The specific oligonucleotide primers of those genes for PCR amplification were designed from sequences deposited within the GenBank database (https://www.ncbi.nlm.nih.gov/) and the right amplicons were confirmed by cloning, sequencing and homology searches against entries within the database using standard nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Each pair of primers used in the qRT-PCR reaction, was designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) by taking into consideration the length of each primer (18–20 bp), melting temperature (55–60 °C), C+G contents (40–66%) and target size (100–210 bp). Then, qRT-PCR was performed on the same RNA samples. The amplification efficiency (E) of each individual gene was calculated according to the Equation 1 provided by Brankatschk et al. (2012):

namely $E = [10^{(-1/slope)} - 1]$ (1)

A standard curve was used to calculate the qRT-PCR amplification efficiency. A series of five-fold dilutions, prepared in triplicate, (200, 100, 50, 25 and 20 ng relative to the original concentration of RNA to the cDNA sample). The correlation coefficient (R^2) value of the slope was used to calculate efficiency. All PCR reactions were conducted in 96-well plates using a StepOnePlus™ Real-Time PCR System (Applied Biosystems; USA). Each gene reaction contained 1× SYBR® master mix (Thermo Fisher Scientific, USA), 0.3 µM of the primers and 1 µL cDNA and used the following qRT-PCR conditions: pre-denaturation at 95°C for 10 min, followed by 40 cycles of amplification of 95°C for 15 secs, 60°C for 45 secs and then finishing with melting curve analysis (95°C for 1 min followed by 60°C and 95°C for 30 secs) to confirm the specific gene products. The threshold cycle (Ct) value for each reaction was exported and analyzed using the StepOne™ software (Applied Biosystems; USA). The qRT-PCR condition of each gene was optimized and then chosen for determining gene expression stability.

Data of gene expression stability analysis

The Ct data obtained from the real-time PCR of each sample were analyzed using three different Microsoft Excel-based software applications: NormFinder (Andersen et al., 2004), Best Keeper (Pfaffl et al., 2004) and a comparative Ct method (Silver et al., 2006).

NormFinder is a statistical modeling approach for evaluating stable reference genes by calculating both the intra- and intergroup variations which are then combined into a stable value (Q) (Andersen et al., 2004). Candidate reference genes with the lowest intragroup and intergroup variations, consequently with the lowest stability values, are the most stable reference genes for use using Equation 2:

$Q = (1+Eff)^{\Delta Ct}$; ΔCt = lowest Ct value of all samples of this gene-Ct value of sample (2)

BestKeeper analyzes the descriptive statistics of the cycle threshold values (Ct) of each gene. The BestKeeper index is calculated as the mean of the candidate reference gene Ct values (Pfaffl et al., 2004). This software ranks stable genes based on a high coefficient of correlation (r) of Ct values to the BestKeeper index. Standard deviations (SD) of the Ct values are also important because genes with a high SD for their Ct values are considered unstable and therefore, not appropriate for use.

The comparative Ct method as detailed by Silver et al. (2006), estimates the stability of gene expression by comparing the relative expression of each sample. This method was used to compare the Ct values between the different groups. The candidate gene with the smallest SD to the ΔCt values was considered the most stable for use.

Relative cyp4 expression using quantitative real-time polymerase chain reaction

The mRNA transcripts of *cyp4* in the digestive glands of green mussels were analyzed to demonstrate the effect of reference gene selection on the interpretation of target gene expression data. Each reaction was performed in triplicate and in a 15 µL final volume as detailed above. The derived threshold cycle (Ct) value for each reaction was then exported and analyzed using the StepOne™ software. The relative expression levels were normalized with the three candidate reference genes using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The data were subjected to one-way analysis of variance followed by Tukey's honest significant difference test. Values of $p < 0.05$ were considered significant.

Ethics statement

This study was approved by the Research Ethics Committee for the Graduate School, Faculty of Science, Burapha University (Approval ID # 15/2558).

Results and Discussion

Amplification specificity and efficiency of the candidate reference genes

Three traditional HKGs (*18S rRNA, 28S rRNA*, *β-actin*) were chosen for this study to evaluate their stability by considering three separate experimental factors (the maturity stage and gender of the green mussel, *P. viridis*, and their response when exposed to BPA). In addition to these, *cyp4* was used as the target gene in qRT-PCR for determining the suitable normalization between each reference gene. *Cyp4* was selected to serve as a biomarker of BPA adulteration in the coastal environment. A summary of the genes, the newly designed primers and their characteristics are provided in Table 1. The specific primers for each gene successfully amplified their respective targets with a single, distinct peak of the melting curve with a T_m (melting temperature) ranging from 77.5°C to 82.3°C, confirming gene-specific amplification (Fig. 2A). A single PCR product of those genes of the expected size (116, 157, 213, 207 bp, respectively) was supported by gel electrophoresis (Fig. 2B). The correlation coefficient (R^2) and the PCR amplification efficiency (E) of each gene within the digestive glands of each pool of mussels were calculated. The results ($R^2 = 0.99$ for all genes; E = 100, 97, 94 and 100% for *18S rRNA, 28S rRNA, β-actin* and *cyp4*, respectively) as seen in Table 1, met the standard mentioned in Ramarkers et al. (2003) ($R^2 > 0.99$, $90 < E\% < 110$).

In this study, the Ct-values in qRT-PCR provided an overview of the gene expression levels, which were measured with each cycle as it approached the necessary exponential phase of the PCR. The higher the Ct value, the lower the level of expression. Among the genes that were tested for the three different conditions, the ribosomal RNA genes (*18S* and *28S*) showed high expression with low Ct values (average Ct values of 15.95 and 10.09, respectively),

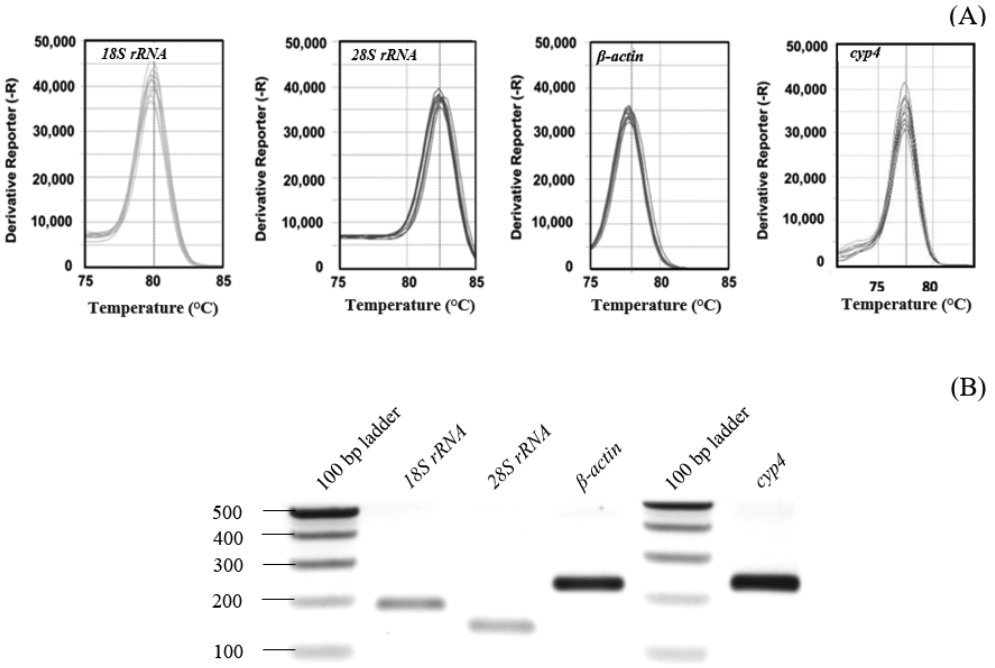

Fig. 2 Confirmation of gene specificity and size of amplicons from green mussel (*Perna viridis*): (A) melting curve analysis of three candidate reference genes and target *cyp4* gene; (B) agarose gel electrophoresis of three amplified candidate reference genes and target *cyp4* gene

Table 1 Characteristics of genes and their primers used for quantitative real-time polymerase reaction in *Perna viridis*.

Gene	Gene description	GenBank accession no.	Primer sequences (5'-3') forward/reverse	Amplicon length (bp)	Melting T_m (°C)	Efficiency (%)	Correlation coefficient (R^2)
18S rRNA	*18S ribosomal RNA*	KY081328	AAGGGCACCACCAGGAGT/ AACCAGACARATCRCTCCAC	157	80	97	0.99
28S rRNA	*28S ribosomal RNA*	JQ622201	CGAAGCCAGAGGAAAATCTG/ GAAACTTCGGAGGGAACCA	116	82.3	100	0.99
β-actin	*β-actin*	EU381234	AGATCTTGCAGGACGTGACC/ GTGATTACTTGCCCGTCAGG	213	78	94	0.99
cyp4	*Cytochrome P450 4*	EU429566	GACCAGTGCAATGACTTGGA/ GACGCCTGTCTACCGATGAT	207	77.5	100	0.99

whereas a cytoskeleton *β-actin* showed low expression (average Ct value of 30.78; Fig. 3). Typically, Ct values between 18 and 30 are considered appropriate and effective levels (Jiang et al., 2014) and all the Ct values obtained in this study were within these guideline values. Since the average Ct value of the *18S rRNA* was closer to the standard range than the others, it was regarded to likely be more appropriate. In addition to this, the Ct values of the ribosomal RNA genes (*18S* and *28S*) showed less variation than *β-actin* (Fig. 3A–C) which was particularly influenced by the difference in developmental status (juvenile and mature stages) as shown in Fig. 3A. However, the abundance of mRNA transcripts with lower Ct values of the ribosomal RNA genes were not surprising as these genes are repeated with 100 to 10,000 copies in cells (Woolford, Jr., 2003).

Stability of candidate reference genes under different conditions

To identify the most suitable reference gene within the digestive glands of green mussels for qRT-PCR analysis considering the three different experimental factors (maturity status, gender, BPA exposure), three software methods were used (BestKeeper, NormFinder and a comparative Ct method) to individually analyze the expression stability of those genes. The software calculated the stability of gene expression and then ranked them, based upon their SD values. The candidate HKGs with Ct values with a small SD identified genes that were more stable. The data from the analysis using the three programs is summarized in Table 2.

NormFinder calculated all the genes tested with SD values lower than 1. This mathematical algorithm ranked the genes in the order *28S rRNA, 18S rRNA*, *β-actin,* respectively, as the best reference gene considering the maturity of green mussel (SD values of 0.188, 0.212, 0.331, respectively; see Fig. 4A) and in the same order considering the gender of green mussel (SD values of 0.234, 0.244, 0.296, respectively; see Fig. 4B), albeit with little difference between the two most stable genes. However, when the exposure to BPA was considered, the rank order of the genes was not the same; *18S rRNA* had the lowest SD value (0.181) among the tested genes. The SD value for *28S rRNA* was 0.196 (Fig. 4C), while that for *β-actin* was the largest (SD of 0.198) and therefore was considered the least appropriate here. However, there was no significant difference among the three genes.

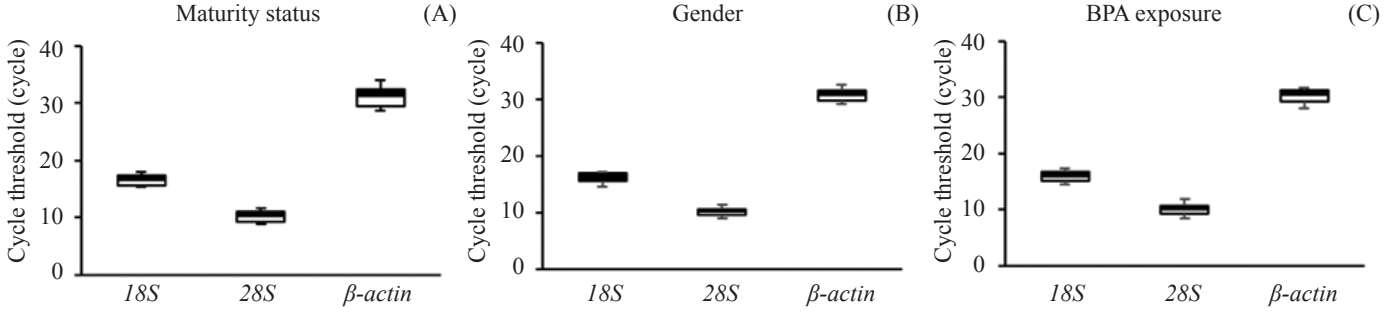

Fig. 3 Quantification cycle threshold value of three candidate reference genes in *Perna viridis* compared by: A) maturity status (juvenile and mature stages); B) gender (♂ and ♀); C) exposure to BPA (0 ng and 10 ng BPA), where the lower and upper boundaries of each box indicate the 25th and 75th percentile, respectively, the horizontal line within the box indicates the median value and fractions above and below the box indicate the 90th and 10th percentile, respectively

Table 2 Gene expression stability ranked using BestKeeper, NormFinder and a comparative threshold cycle (Ct) method

Factor	Rank	BestKeeper		NormFinder		Comparative Ct	
		Gene	SD	Gene	Stability	Gene	SD
Maturity	1	*28S rRNA*	0.843	*28S rRNA*	0.188	*28S rRNA*	1.943
	2	*18S rRNA*	0.903	*18S rRNA*	0.212	*18S rRNA*	1.984
	3	*β-actin*	1.745	*β-actin*	0.331	*β-actin*	2.333
Gender	1	*28S rRNA*	0.647	*28S rRNA*	0.234	*28S rRNA*	0.338
	2	*18S rRNA*	0.832	*18S rRNA*	0.244	*18S rRNA*	0.346
	3	*β-actin*	1.072	*β-actin*	0.296	*β-actin*	0.450
BPA exposure	1	*28S rRNA*	0.832	*18S rRNA*	0.181	*28S rRNA*	0.392
	2	*18S rRNA*	0.845	*28S rRNA*	0.196	*18S rRNA*	0.425
	3	*β-actin*	1.117	*β-actin*	0.198	*β-actin*	0.463

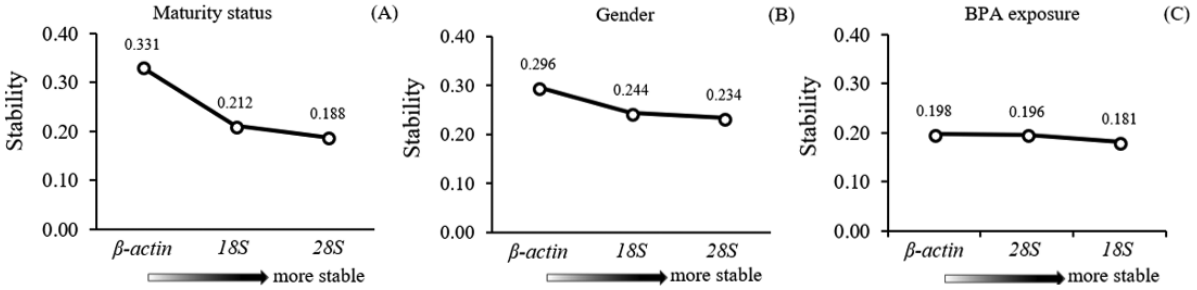

Fig. 4 Stability of candidate reference genes using NormFinder (from least stable to more stable) compared by: A) maturity status (juvenile and mature stages); B) gender (♂ and ♀); C) exposure to BPA (0 ng and 10 ng BPA), where gene stability is determined by its threshold cycle value, for which a gene with a small SD is more stable

The results from the application of BestKeeper agreed with the comparative Ct method in that the *28S rRNA* gene was the best performer under each of the tested conditions, followed by the *18S rRNA* gene and the *β-actin* gene was the least suitable. Box-and-whisker plots with SD value ranges for each are presented in Fig. 5 (BestKeeper) and Fig. 6 (comparative Ct method). However, no significant difference was observed among the three genes under each individually tested condition. It was noted that SD values of the BPA exposed groups within the first two conditions were very similar (0.832/0.845 from the BestKeeper and 0.392/0.425 from the comparative Ct method). Therefore, it appeared that when using qRT-PCR to analyze green mussels of differing maturity stages or gender or both, the ribosomal RNA genes are more appropriate to be used as an endogenous control than cytoskeletal *β-actin.* A similar pattern of results has been reported for many mollusc species, including *P. viridis*, *M. edulis* and *Bellamya aeruginosa* (see the various works of Lui and Kueh, 2005; Cubero-Leon et al., 2011; Cubero-Leon et al., 2012; Miao et al., 2014; Lui et al., 2016), as well as in various vertebrate species, such as *Catla catla, Oryzias latipes, Taeniopygia guttata, Gallus gallus* f. *dom.* (see the studies of Zhang and Hu, 2007; Olias et al., 2014; Faheem et al., 2018). In addition to this and in support of the findings in this study, Feng et al. (2013) reported that *β-actin* was the least stable gene to use when analyzing the larvae and adults of the Yesso scallop, *Patinopecten yessoensis,* when exposed to phenanthrene at different salinities.

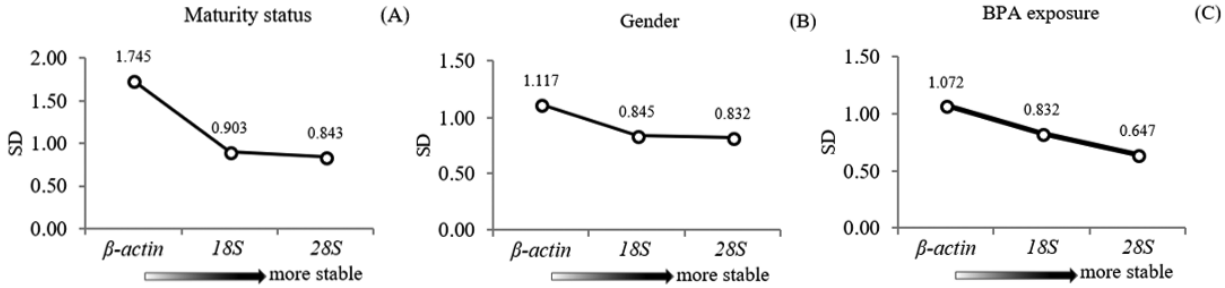

Fig. 5 Stability of the candidate reference genes using BestKeeper (from least stable to most stable) compared by: A) maturity status (juvenile and mature stages); B) gender (♂ and ♀); and, C) exposure to BPA (0 ng and 10 ng BPA). A gene's stability is determined by its threshold cycle value, for which a gene with a small SD is more stable

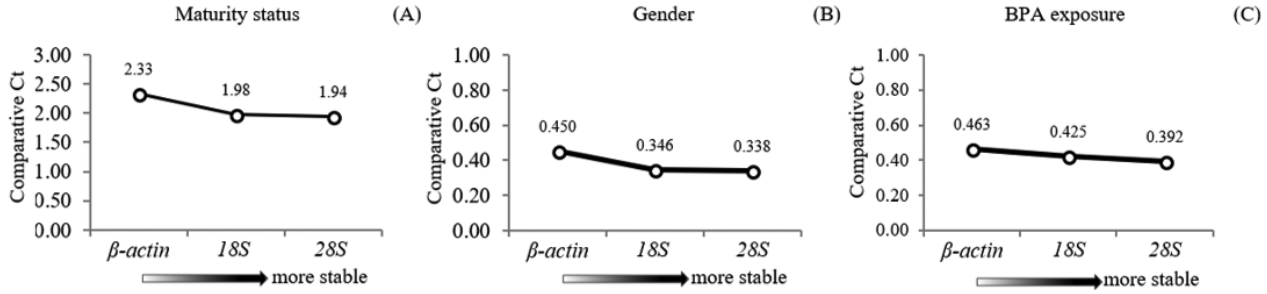

Fig. 6 Stability of the candidate reference genes using a comparative threshold cycle (Ct) method (from least stable to most stable) compared by: A) maturity status (juvenile and mature stages); B) gender (♂ and ♀); and, C) exposure to BPA (0 ng and 10 ng BPA). A gene's stability is determined by its Ct value, where a gene with a small SD is more stable

Relative expression levels of cytochrome P450 4 (cyp4) using quantitative real-time polymerase chain

For qRT-PCR to be accurate for the biomonitoring of harmful BPA contamination, appropriate internal reference genes for normalization must be determined. Generally, the expression of the reference gene should be minimally changed, while the target gene should vary over the course of the experiment. The expression levels of the target gene should also vary between different tissues and environmental conditions. Therefore, in the current study, the *cytochrome P450 4* gene or *cyp4* was selected as a potential biomarker to evaluate changes in expression in the digestive glands of green mussels when exposed to BPA.

In bivalves, the digestive gland plays a central role like that of the liver in vertebrates, in the metabolism and detoxification of xenobiotic agents (Cocci et al., 2017; Rodrigo and Costa, 2017). *Cyp4*, the target gene in this study, encodes the CYP4 enzyme which is mainly involved in xenobiotic metabolisms (Hardwick, 2008; Lewis and Ito, 2008). *Cyp4* has been seen in several bivalves, such as *P. viridis*, *Modiolus modiolu, Mytilus edulis* and *M. galloprovincialis* (Jonsson et al., 2006; Veldhoen et al., 2009; Ravlić et al., 2015), and also in marine invertebrates such as the polychaete *Perinereis aibuhitensis,* which responds to many pollutants (Chen et al., 2012; Leung et al., 2014). The *CYP4* gene in humans has also been reported to be involved in xenobiotic and pharmaceutical metabolism (Kalsotra and Strobel, 2006).

In the current study, the results for the three candidate reference genes that were individually normalized for the *cyp4* mRNA transcripts, showed similar relative expression. All the expressions were down-regulated in green mussels when exposed to 10 ng BPA for 12 hr and compared to the unexposed control group, as seen in Fig. 7. The decreasing levels of *cyp4* expression differed between the internal reference genes used (0.549, 0.112 and 0.456-fold changes for *18S rRNA*, *28S rRNA* and *β-actin*, respectively) were also noted. However, BPA significantly decreased the expression levels of *cyp4* only when *18S rRNA* was normalized. This result was in accordance with the findings reported by Jonsson et al. (2006), who exposed larvae of *Chironomus riparius* to 0.5 mgL^{-1} BPA and observed a subsequent down-regulation in *cyp4* expression. The results of the current study indicated that the *18S rRNA*, a commonly used housekeeping gene, was the most appropriate reference gene for use in qRT-PCR with the green mussel (*P. viridis*) independent of their maturity or gender status and serves as an appropriate monitoring method for BPA contamination.

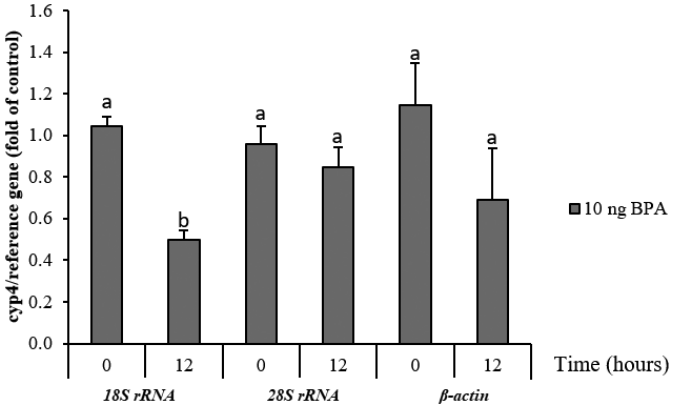

Fig. 7 *cyp4* expression levels (mean ± SD) of mature *Perna viridis* injected with 10 ng BPA exposed for 0 hr and 12 hr, where changes in expression levels were analyzed using quantitative real-time polymerase chain reaction with normalization against different housekeeping genes, , different lowercase letters above bars indicate significant difference ($p < 0.05$).

In the current study, the stability was explored of three traditionally used housekeeping genes (*18S rRNA*, *28S rRNA*, *β-actin*) in green mussels under different experimental conditions and analyzed using qRT-PCR analysis. Although the gene expression profiles were variable, the most suitable reference gene was carefully selected and tested for normalization for use within a sensitive quantitative real-time PCR assay. Of the genes tested, *18S rRNA* within the digestive gland of *P. viridis* was the most appropriate reference gene for use. Its selection was derived from its expression stability following analyses exploring mussel gender, maturity stage and under conditions of BPA exposure. However, *β-actin* was the least appropriate reference gene for use in this situation. From the experimental data and notably the derived mRNA transcripts, *P. viridis* appeared to represent an appropriate species for the biomonitoring of BPA exposure in marine waters.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors gratefully acknowledge the Department of Aquatic Science, Burapha University, Chonburi, Thailand for use of the wet laboratory facilities for the purposes of this study. In addition, the authors thank Mr Akkarat Noipeng for the maintenance of the green mussels throughout the experimental trials.

References

Andersen, C.L., Jensen, J.L., Ørntoft, T.F. 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 64: 5245–5250.

Boonyatumanond, R., Jaksakul, A., Puncharoen, P., Tabucanon, M.S. 2002. Monitoring of organochlorine pesticides residues in green mussels (*Perna viridis*) from the coastal area of Thailand. Environ. Pollut. 119: 245–252.

Brankatschk, R., Bodenhausen, N., Zeyer, J., Bürgmann, H. 2012. Efficiency of real-time qPCR depends on the template: a simple absolute quantification method correcting for qPCR efficiency variations in microbial community samples. Appl. Environ. Microbiol. 78: 4481–4489.

Bustin, S.A. 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): Trends and problems. J. Mol. Endocrinol. 29: 23–39.

Canesi, L., Fabbri, E. 2015. Environmental effects of BPA: focus on aquatic species. Dose Response 13: 1–14.

Chen, X., Zhou, Y., Yang, D., Zhao, H., Wang, L., Yuan, X. 2012. CYP4 mRNA expression in marine polychaete *Perinereis albuhitensis* in response to petroleum hydrocarbon and deltamethrin. Mar. Poll. Bull. 64: 1782–1788.

Chouhan, S., Yadav, S.K., Prakash, J., Swati, Surya, S. 2014. Effect of bisphenol A on human health and its degradation by microorganisms: A review. Ann. Microbiol. 64: 13. doi: https://doi.org/10.1007/s13213-013-0649-2.

Cocci, P., Capriotti, M., Mosconi, G., Palermo, F.A. 2017. Transcriptional variations in biomarkers of *Mytilus galloprovincialis* sampled from Central Adriatic coastal waters (Marche Region, Italy). Biomarkers. 22: 1–28. doi:10.1080/1354750X.2017.1315614.

Cubero-Leon, E., Ciocan, C.M., Minier, C., Rotchell, J.M. 2011. Reference gene selection for qPCR in mussel, *Mytilus edulis*, during gametogenesis and exogenous estrogen exposure. Environ. Sci. Pollut. Res. Int. 19: 2728–2733.

Cubero-Leon, E., Puinean, A.M., Labadie, P. et al. 2012. Two CYP3A-like genes in the marine mussel *Mytilus edulis*: mRNA expression modulation following short-term exposure to endocrine disruptors. Mar. Environ. Res. 74: 32–39.

Dermitzakis, E. 2008. Regulatory variation and evolution: Implications for disease. Adv. Genet. 61: 295–306.

Faheem, M., Jahan, N., Khaliq, S., Lone, K.P. 2018. Validation of reference genes for expression analysis in a teleost fish (*Catla catla* Hamilton) exposed to an endocrine-disrupting chemical, bisphenol-A. Rendiconti Lincei. Scienze Fisiche e Naturali. 29: 13–22.

Feng, L., Yu, Q., Li, X., et al. 2013. Identification of reference genes for qRT-PCR analysis in Yesso scallop *Patinopecten yessoensis*. PLoS ONE. 8: e75609. doi:10.1371/journal.pone.0075609

Flint, S., Markle, T., Thompson, S., Wallace, E. 2012. Bisphenol A exposure, effects, and policy: A wildlife perspective. J. Environ. Manage. 104: 19–34.

Fung, C.N., Lam, J.C.W., Zheng, G.J., Connell, D.W., Monirith, I., Tanabe, S., Richardson, B.J., Lam, P.K.S. 2004. Mussel-based monitoring of trace metal and organic contaminants along the east coast of China using *Perna viridis* and *Mytilus edulis*. Environ. Pollut. 127: 203–216.

Hardwick, J.P. 2008. Cytochrome P450 omega hydroxylase (CYP4) function in fatty acid metabolism and metabolic diseases. Biochem. Pharmacol. 75: 2263–2275.

Isobe, T., Takada, H., Kanai, M., Tsutsumi, S., Isobe, K.O., Boonyatumanond, R., Zakaria, M.P. 2007. Distribution of polycyclic aromatic hydrocarbons (PAHs) and phenolic endocrine disrupting chemicals in South and Southeast Asian mussels. Environ. Monit. Assess. 135: 423–440.

Jiang, Q., Wang, F., Li, M.Y., Tan, G.F., Xiong, A.S. 2014. Selection of suitable reference genes for qPCR normalization under abiotic stresses in *Oenanthe javanica* (Bl.) DC. PLoS ONE 9: e92262. https://doi.org/10.1371/journal.pone.0092262

Jonsson, H., Schiedek, D., Grøsvik, B.E., Goksøyr, A. 2006. Protein responses in blue mussels (*Mytilus edulis*) exposed to organic pollutants: a combined CYP-antibody/proteomic approach. Aquat. Toxicol. 78: S49–S56.

Kalsotra, A., Strobel, H.W. 2006. Cytochrome P450 4F subfamily: At the crossroads of eicosanoid and drug metabolism. Pharmacol. Ther. 112: 589−611.

Leung, P.T.Y., Ip, J.C.H., Mak, S.S.T., Qiu, J.W., Lam, P.K.S., Wong, C.K.C., Chan, L.L., Leung, K.M.Y. 2014. *De novo* transcriptome analysis of *Perna viridis* highlights tissue-specific patterns for environmental studies. BMC Genomics. 15: 804. doi: 10.1186/1471-2164-15-804

Lewis, D.F.V., Ito, Y., 2008. Cytochrome P450 structure and function: an evolutionary perspective. In: Ioannides, C. (Ed.). Cytochromes P450. Role in the Metabolism and Toxicity of Drugs and Other Xenobiotics. Issues in Toxicology. RSC Publishing, Cambridge. pp. 3–45.

Livak, K.J, Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2[-Delta Delta C(T)] method. Methods. 25: 402–408.

Lui, J.H., Kueh, C.S.W. 2005. Biomonitoring of heavy metals and trace organics using the intertidal mussel *Perna viridis* in Hong Kong coastal waters. Mar. Pollut. Bull. 51: 857–875.

Lui, Q., Lei K., Ma, Q., Qiao, F., Li, Z. An, L. 2016. Ribosomal protein L7 as a suitable reference gene for quantifying gene expression in gastropod *Bellamya aeruginosa.* Environ. Toxicol. Pharmacol. 43: 120–127.

Maier, T., Güell, M., Serrano, L. 2009. Correlation of mRNA and protein in complex biological samples. FEBS Lett. 583: 3966–3973.

Mathieu-Denoncourt, J., Wallace, S.J., de Solla, S.R., Langlois, V.S. 2015. Plasticizer endocrine disruption: Highlighting developmental and reproductive effects in mammals and non-mammalian aquatic species. Gen. Comp. Endocrinol. 219: 74–88.

Miao, J., Pan, L., Zhang, W., Liu, D., Cai, Y., Lia, Z. 2014. Identification of differentially expressed genes in the digestive gland of Manila clam *Ruditapes philippinarum* exposed to BDE-47. Comp. Biochem. Physiol. C. 161: 15–20.

Monirith, I., Ueno, D., Takahashi, S. et al. 2003. Asia-Pacific mussel watch: monitoring contamination of persistent organochlorine compounds in coastal waters of Asian countries. Mar. Pollut. Bull. 46: 281–300.

Ocharoen, Y., Boonphakdee, C., Boonphakdee, T., Shinn, A.P., Moonmangmee, S. 2018. High levels of the endocrine disruptors bisphenol-A and 17β -estradiol detected in populations of green mussel, *Perna viridis*, cultured in the Gulf of Thailand. Aquaculture. 497: 348–356.

Oehlmann, J., Schulte-Oehlmann, U., Bachmann, J., Oetken, M., Lutz, I., Kloas, W., Ternes T.A. 2006. Bisphenol A induces superfeminization in the ramshorn snail *Marisa cornuarietis* (Gastropoda: Prosobranchia) at environmentally relevant concentrations. Environ. Health Perspect. 114: 127–133.

Olias, P., Adam, I., Meyer, A., Scharff, C., Gruber, A.D. 2014. Reference genes for quantitative gene expression studies in multiple avian species. PLoS ONE. 9: e99678. doi: 10.1371/journal.pone.0099678.

Pfaffl, M.W., Tichopad, A., Prgomet, C., Neuvians, T.P. 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. Biotechnol. Lett. 26: 509–515.

Ramarkers, C., Ruijter, J.M., Deprez, R.H.L., Moorman, A.F.M. 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. 339: 62–66.

Ravlić, S., Žučko, J., Tanković, M.S., Fafanđel, M., Bihari, N. 2015. Sequence analysis of novel CYP4 transcripts from *Mytilus galloprovincialis*. Environ. Toxicol. Pharmacol. 40: 300–309.

Rodrigo, A.P., Costa, P.M. 2017. The role of the cephalopod digestive gland in the storage and detoxification of marine pollutants. Front. Physiol. 8: 232. doi: 10.3389/fphys.2017.00232.

Ruangwises, N., Ruangwises, S. 1998. Heavy metals in green mussels (*Perna viridis*) from the Gulf of Thailand. J. Food Prot. 61: 94–97.

Salgueiro-González, N., Concha-Graña, E., Turnes-Carou, I, Muniategui-Lorenzo, S., López-Mahía, P., Prada-Rodríguez, D. 2012. Determination of alkylphenols and bisphenol A in seawater samples by dispersive liquid-liquid microextraction and liquid chromatography tandem mass spectrometry for compliance with environmental quality standards (Directive 2008/105/EC). J. Chromatogr. A. 1223: 1–8.

Schettino, T., Caricato, R., Calisi, A., Giordano, M., Lionetto, M. 2012. Biomarker approach in marine monitoring and assessment: new insights and perspectives. Open Environ. Sci. 6. doi: 10.2174/1876325101206010020.

Silver, N., Best, S., Jiang, J., Thein, S.L. 2006. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. BMC Mol. Biol. 7: 33. doi: 10.1186/1471-2199-7-33

Veldhoen, N., Lowe, C.J., Davis, C., Mazumder, A., Helbing, C.C. 2009. Gene expression profiling in the deep water horse mussel *Modiolus modiolus* (L.) located near a marine municipal wastewater outfall. Aquat. Toxicol. 93: 116–124.

Wong, M.L., Medrano, J.F. 2005. Real-time PCR for mRNA quantitation. Biotechniques. 39: 75–85.

Woolford Jr., J.L. 2003. Ribosome assembly. In: Lennarz, W.J., Lane, M.D. (Eds.). Encyclopedia of Biological Chemistry. Academic Press. CA, USA. pp. 719–724.

Xue, J., Kannan, K. 2016. Novel finding of widespread occurrence and accumulation of bisphenol A diglycidyl ethers (BADGEs) and novolac glycidyl ethers (NOGEs) in marine mammals from the United States coastal waters. Environ. Sci. Technol. 50: 1703–1710.

Zhang, Z., Hu, J. 2007. Development and validation of endogenous reference genes for expression profiling of Medaka (*Oryzias latipes*) exposed to endocrine disrupting chemicals by quantitative real-time RT-PCR. Toxicol. Sci. 95: 356–368.