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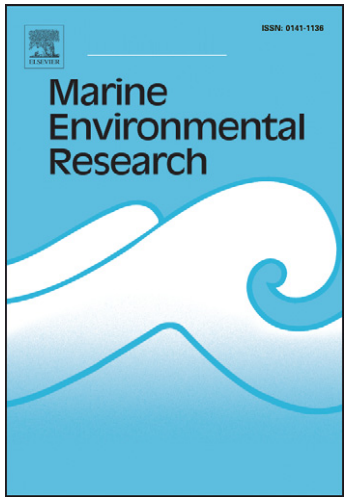

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Molecular cloning of a new member of the p53 family from the Pacific oyster *Crassostrea gigas* and seasonal pattern of its transcriptional expression level.

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ABSTRACT

Like other sessile filter-feeding molluscs, oysters may be exposed in the natural environment to a variety of contaminants. Long-term exposure to pollutants may be one factor affecting prevalence of cancerous-like disorders, such as neoplasia. Environmentally induced alterations in p53 protein expression, in relation to leukemia, have been reported in various mollusc species inhabiting polluted water, suggesting that p53 proteins can also be used as a marker for environmental research. This work reports the cloning and sequencing of a p53-like cDNA in the mollusc bivalve *Crassostrea gigas*. The deduced amino acid sequences of p53 shared a high degree of homology with the homologues from other mollusc species, including typical eukaryotic p53 signature sequences. We examined the p53 transcription expression pattern during the annual cycle in oyster gills and whole soft tissues in four locations along the French coasts. Real-time PCR analysis suggested that strong variations at p53 mRNA level are probably synchronized with the seasonal cycle at the four locations investigated.

KEYWORDS

***Ecotoxicology, *Oyster, *mRNA, p53, seasonal variations, cell stress marker**

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1. INTRODUCTION

The oyster *Crassotrea gigas* is widely used in ecotoxicology and environmental monitoring. The sessile nature of this species facilitates the establishment of cause-and-effect relationships in time and space and minimizes the possible confounding factors associated with the use of migratory species. As filter-feeding molluscs, oysters are potentially exposed to a variety of pollutants. Long-term exposure to contaminants may possibly compromise immune function, progressively lead to infectious diseases and may be one factor affecting prevalence of "cancerous-like" disorders such as neoplasia (Smolarz et al., 2005). Cases of haemic neoplasia (or leukemia) have been described in bivalve molluscs (Moore et al., 1991; Moore and Elston, 1991), especially in *Mya arenaria* (Leavitt et al., 1990; Kelley et al., 2001; Stephens et al., 2001; Barber, 2004) and *Mytilus edulis* (Noel et al., 1994). Several studies suggest the implication of p53 and its family members in this disease. Mutations in the p53 transcript were reported in leukemic *vs.* normal hemocytes of *M. arenaria* (Barker et al., 1997). In addition, p73 protein is up-regulated in hemocytes from leukemic *M. arenaria* (Kelley et al., 2001).

In mammals, the protein p53 was shown to play a central role in the supervision of genome integrity. Indeed, p53 plays several major roles in cell cycle arrest, DNA repair, apoptosis and carcinogenesis. In human, mutations of the gene p53 are observed in about a half of cancer cases and in tumors where the gene is not mutated, an alteration of the post-translational regulation of the protein is frequently observed (O'Brate and Giannakakou, 2003; May & May, 1999 for a review). This modification of p53 jeopardizes the apoptosis that would normally occur when cellular DNA damage cannot be repaired.

Due to its central role in cell cycle regulation and in the response to cellular stress, members of p53 family (p63/73) may be used as markers of cellular stress in the mussel (St-Jean et al., 2005). The first molluscan p53 to be identified originated from the squid *Loligo forbesi* (Ishioka et al., 1995) but the squid sequence is more similar to p63/73, close homologues of p53. Sequences belonging to the p53 family have now been identified in various bivalve molluscs, showing evidence of their ubiquitous existence in this class: *Spisula solidissima* (Jessen-Eller et al., 2002), *Mya arenaria* (Kelley et al., 2001), *Mytilus galloprovincialis* (Dondero et al., 2006), *Mytilus trossulus* (Muttray et al., 2005; Muttray et al., 2007), *Mytilus edulis* (Ciocan & Rotchell, 2005; Muttray et al., 2005; Muttray et al., 2007), *Crassostrea*

rhizophorae (a partial coding sequence, unpublished). This paper reports the cloning of a cDNA encoding a p53 homologue in the pacific oyster *Crassostrea gigas*.

In the natural environment, oyster physiology (reproduction, growth, immunity) is known to be strongly driven by the seasonal cycle (Samain & Mc Combie, 2007). In this study, real-time polymerase chain reaction (or quantitative PCR) was used to monitor the potential seasonal changes of p53 mRNA level in comparison with mRNA encoding for actin, as a reference gene. Samples were collected monthly and quarterly on the French coast on the Atlantic Ocean and the English Channel in order to build time series measurements of gene expression. Total RNA was extracted from whole oyster soft part homogenates as well as from isolated gills. Statistical analysis of these data was performed, first to characterize the ranges of fluctuations of p53 mRNA level in the natural environment, as well as possible seasonal variations, and secondly, to compare different populations along the French coast.

2. MATERIALS AND METHODS

2.1 p53 cDNA cloning

2.1.1 Search for a specific fragment using degenerate oligonucleotides

Oysters *Crassostrea gigas* were obtained from oyster farms located at four different sites (Carnac, Cancale, Asnelles, St-Vaast, see Figure 1). Attention was paid to ensure that all of the oysters were diploid, 3-4 years-old, had spent their entire growing life in the farm and had been removed from seawater a few minutes before sampling. Total RNA was extracted from isolated gills using TRI REAGENT (Sigma-Aldrich) and used for purification of polyadenylated-RNA (Dynabeads® mRNA DIRECT Kit from Dynal Biotech). Poly-A RNA (100 ng) was reverse-transcribed using 150 ng oligo(dT) primer, 300 U moloney murine leukemia virus (MMLV) reverse transcriptase (Sigma-Aldrich), RNAse free dNTP and 40 U RNAsin (Promega).

The cDNA obtained was used for polymerase chain reaction (PCR) cycling with degenerate primers *deg_p53_F and deg_p53_R* (Table 1 and Figure 2). These degenerate oligonucleotides were designed on the basis of conserved amino acids of p53 from various eukaryotic organisms. The primers were purchased from EUROBIO. The PCR was performed with 50µl of a mixture containing 5µl of 10X buffer (Tris–HCl 100 mM, KCl 500 mM and

1.0% Triton® X-100, Promega), 4 mM $MgCl_2$, 0.2 mM of each dNTP, 1 U of *Taq* DNA polymerase (Promega), 1 µM of each degenerate primer and 20 ng cDNA, reverse-transcribed from poly-A RNA. This mixture was subjected to 40 cycles of amplification (denaturation for 30 sec at 95 °C, annealing for 30 sec at 45 °C and extension for 30 sec at 72 °C).

The PCR products were separated on a Tris-Acetate-EDTA (TAE) 1% agarose gel. A single band of approximately 350 bp was obtained. This PCR product was purified (Wizard SV gel and PCR clean-up system, Promega), and inserted into the PCR®II Topo® vector (TOPO TA cloning kit, Invitrogen) which was used to transform XL1-blue MRF' competent cells. The p53 cDNA fragment insert was sequenced (both strands) by Genome Express.

2.1.2 Amplification of 5' and 3' ends by RACE PCR

In order to determine the missing sequence of 5′ and 3' cDNA ends, a RACE-PCR procedure was performed using the GeneRacer RACE Ready cDNA Kit (Invitrogen) and following manufacturer's instructions. Double-stranded cDNA reverse-transcribed from poly-A RNA was ligated to adaptors, and 6 ng of this template was used to amplify 5'- and 3'- fragments using adaptor-specific primers and gene-specific primers (Table 1) deduced from the initial 350 bp fragment sequence. The proof-reading Platinum Taq DNA polymerase High Fidelity (Invitrogen) was used for "touchdown" PCR with the following cycling conditions: 2 min at 94 °C, followed by 5 cycles each of 30 s at 94 °C and 1 min at 72 °C, followed by 5 cycles each of 30 s at 94 °C and 1 min at 70 °C, followed by 25 cycles each of 30 s at 94 °C, 30 sec at 65 °C and 1 min at 68°C, with a final extension at 68 °C for 10 min. The 5' RACE PCR procedure led to the amplification of two bands of c.a. 900 bp and 700 bp. The 3' RACE PCR procedure led to only one band of c.a. 1100 bp. These three bands were purified (Wizard SV gel and PCR clean-up system, Promega), cloned and sequenced as described previously. The 700 bp sequence obtained from the 5' RACE procedure was not related to p53 and was discarded.

2.1.3 Amino acid sequence analysis

In order to search for homologues, the deduced amino-acid sequence from *Crassostrea gigas* was submitted to BLAST2 (http://expasy.org/tools/blast/). To illustrate conserved protein domains, pairwise amino acid alignment of the deduced *Crassostrea gigas* p53 protein was performed with other available full-length p53 from molluscan and human species using

ClustalW (1.83) multiple sequence alignment (http://www.ebi.ac.uk/clustalw/; residue substitution matrix Blosum30) (Higgins et al., 1994) and the software Bioedit Sequence Alignment Editor v.7.0.4.1 (Hall, 1999). Gap opening penalty was set to 25; gap extension penalty and gap separation penalty were set by default. Homology scores (%) were generated from this alignment.

2.2 p53 mRNA expression analysis: seasonal variation and tissue distribution

2.2.1 Sampling

Animals were collected from oyster farms located at the four sampling sites, as described in section 2.1.1. Oysters were collected monthly in St-Vaast and quarterly in Asnelles, Cancale and Carnac between January 2004 and September 2005 (Figure 1). At these four locations, the gills of 20 oysters and the whole soft parts of another 20 oysters were immediately dissected after sampling and frozen in liquid nitrogen. Back in the laboratory, the oyster tissues were crushed in a ball crusher (Verder MM301), in buckets cooled in liquid nitrogen. After this step, five individuals were pooled together as one. The digestive gland, labial palps, posterior adductor muscle, edge of the mantle, gills and hemolymph from 6 oysters collected in St-Vaast in February 2005 were dissected in order to study the relative level of p53 mRNA among different organs. After removing the shell by severing the adductor muscle, hemolymph was withdrawn directly from the pericardial cavity by puncture with a 1 mL sterile syringe equipped with a needle (0.9 × 25 mm). For each oyster, 0.5 mL of hemolymph was withdrawn without any buffer and conserved on ice during collection to prevent hemocytes aggregation. The samples were combined into 2 pools of 3 oysters each to provide sufficient haemocytes for analysis. The gonad was not investigated since it was not clearly distinguishable at this time of the year.

2.2.2 RNA extraction

Total RNA was extracted with TRI REAGENT according to the manufacturer's instructions (Sigma-Aldrich). The amount and quality of RNA was quantified by measurement of absorbance at 260 and 280 nm in a UV-spectrophotometer. To eliminate possible trace of genomic DNA, 550 ng total RNA (nucleic acid extract, strictly speaking) was digested with 1U DNAse I Amplification Grade (Sigma-Aldrich) and an aliquot of DNAse-treated RNA

was then tested by real time PCR with actin primers to test for absence of genomic DNA.

2.2.3 Real- time PCR and Analysis

Reverse transcription (RT) was carried out using 500 ng of total RNA treated with DNAse I, 500 ng random primers (Promega), 200 U Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Promega), 12.5 µmoles RNAse-free dNTP, and 25 U recombinant RNAsin (Promega). The sequences of the forward and reverse primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and synthesized by Eurogentec. For each pair of primers, the efficiency (E) of the reaction was determined by constructing standard curves generated using serial dilutions of template, and using the formula : $E= 100 * (10^{(-1/\text{slope of the standard curve})}-1)$. A slope of –3.32 gives an efficiency of 100%. Primer pairs showing a good efficiency (100 ± 5%) on a range of cDNA concentrations of four orders of magnitude were kept for quantification. The primers used for p53 mRNA quantification by real time PCR were p53_QS for the forward primer and p53_QAS for the reverse primer (Table 1 and Figure 2).

Real-time PCR was performed in a MiQ Cycler (Biorad). All determinations were carried out in duplicates. Controls of no template cDNA were included in PCR experiments. Amplification was carried out in 96-well plates, in a total volume of 15µl containing 7.5µl of 2X iQ SYBR® Green supermix (Biorad), each primer (500 nM final) and cDNA samples obtained from reverse transcription of 5 ng of DNase I-treated total RNA. Amplification conditions were 40 cycles of 15 sec at 95°C and 45 sec at 60°C, followed by the protocol for the melting curve: 80 cycles of 10 sec with an increase of 0.5°C between each cycle from 55°C to 95°C. The specificity of the reaction was confirmed by observing a single peak at the expected Tm on the melting curve analysis and by sequencing the PCR product once (sub-cloning as described in 2.1.1).

2.2.4 Normalization of mRNA expression

Quantification of p53 mRNA was performed by normalization to a reference RNA, using the usual Delta Cycle threshold (Ct) method : normalized p53 mRNA level = $2^{[Ct\ ref\ -\ Ct\ p53]}$. A

preliminary task was to specifically address the choice of the reference gene to express quantitatively gene expression. In order to check the influence of the reference gene, p53 mRNA quantities were normalized to three reference RNAs: actin mRNA (Genbank #AF026063), GAPDH mRNA (Genbank #AJ544886) and 18S ribosomal RNA (Genbank #AB064942), which accounted for total RNA. The annual expression patterns of these reference RNA in oyster were previously investigated by Farcy et al., (2007) and the sequences of these primers are given in Table 1. The seasonal expression pattern of p53 mRNA was found very similar when normalized to these three classically used reference RNA (data not shown) and p53 expression level was finally normalized to actin mRNA. Furthermore the actin Ct value in the range [17.2-19.4] was used to confirm that the starting amount of cDNA was reproducible in all PCR quantifications.

2.2.5 Statistical analysis

Statistical analysis was carried out using Statgraphics Centurion XV (Statpoint, Inc.). For seasonal monitoring of p53 mRNA in oysters from the natural environment, values were expressed as means ± standard deviation of four pooled oyster extracts consisting of five individuals each. For the analysis of the mRNA distribution in organs, values were expressed as means ± standard deviation of six individuals, except for hemolymph (two pools of three individuals). As the hypothesis of normality was rejected by a Shapiro Wilk's W test, data were log-transformed. After this transformation, the hypothesis of homogeneity of variances was accepted using a Bartlett test. Statistically significant differences between two samples were determined using a t-test; * $p<0.05$ was accepted as significant. Multiple sample comparisons were performed using ANOVA. Significance was set at $p<0.05$. Difference between months or between organs was established using the Student Newman Keuls post-hoc test ($p<0.05$). The Newman-Keuls test makes pairwise comparisons of group means after ANOVA has rejected the null hypothesis.

3. RESULTS

3.1 p53 cloning in Crassostrea gigas

The complete procedure for cloning and sequencing was repeated three times independently and led to the identification of one sequence. The RACE procedure for the extension of the 3' cDNA end did not allow the discrimination of isoforms since only one band was found. The full-length cDNA sequence of Cg-p53 is available in the European Molecular Biology Laboratory (EMBL) databank (GenBank accession no. AM236465). The cDNA is 2116 bp long with a 1305 bp open reading frame which encoded a predicted 434 amino acid polypeptide with a calculated molecular mass of 49 kDa protein. This molecular mass is within the range of previously reported p53 proteins. Nucleic acid sequence analysis revealed the presence of typical ATG start and TGA stop codons, a putative ATTAAA polyadenylation signal upstream the polyadenine stretch in the 3' UTR.

The alignment performed between the deduced amino acid sequence of *C. gigas* p53 and other complete sequence of p53 identified in molluscs as well as human is shown in Figure 2. This Clustal Wallis alignment indicated that *Crassostrea gigas* p53 protein shares high homology with other identified homologues from molluscs (Table 2). The p53 protein from oyster possessed 67% to 71% global homology with other molluscan p53 and 29% global homology with human p53.

The deduced amino acid sequences of Cg-p53 exhibited the characteristic domains of the p53 family: 1 - The transactivation domain within its amino terminus including a well-conserved sequence (TAD, Figure 2) and conserved hydrophobic residues involved in MDM2 interaction in human (▲, Figure 2). 2 - a proline-rich region, containing two copies of the PxxP motif. 3 – the central region with the presence of four sequence-specific DNA binding sites (DBD II to DBD V, Figure 2). This central region is the best conserved region of the protein. In the region going from DBD II to DBD IV, the deduced amino acid sequence from oyster is 40% homologous with human p53 and 91% homologous with *Mytilus spp* p53. 4 - the C-terminal region is characterized by a tetramerization domain involved in facilitating the specific binding between p53 and DNA (TD, Figure 2). In that region, a conserved nuclear export signal (NES, Figure 2), as well as two conserved nuclear localization signal sequences (NLS, Figure 2), are present. These functional characteristic regions of the p53 protein family are well conserved between the oyster and human.

3.2 Expression profile of p53 mRNA in Crassostrea gigas tissue

The tissue distribution of p53 mRNA was investigated by real time PCR analysis in a range of *Crassostrea gigas* tissue, sampled in February 2005 (Figure 3). At this time, the gonad is immature and not clearly distinguishable from the digestive gland. For that reason, the gonad was not investigated but we cannot rule out the presence of few gonadic cells in the digestive gland extract. This study showed that p53 mRNA was ubiquitously expressed. The p53 mRNA level is higher (around 2 times) in the posterior adductor muscle, the edge of the mantle and hemolymph, while it was present at lower levels in gills, the digestive gland and labial palps.

3.3 Seasonal variations of p53 transcriptional expression

Monthly recorded values of p53 mRNA levels in gills and whole soft parts in oysters from St-Vaast between January 2004 and September 2005 are shown in figure 4. Animals collected monthly in St-Vaast showed seasonal fluctuations in mRNA level in both isolated gills and whole soft parts. Months when seawater temperature was below 10°C are highlighted as shaded bands in figure 4. It appeared that the p53 mRNA level peaked in the winter period: January-February-March 2004 and February-March-April 2005. A t-test was performed on pairs of maximal (February 2004, March 2005) and minimal (September 2004 and 2005) values and between maximal summer values (July 2004, June 2005) and minimal values. In figure 4, paired values were linked with a solid line in whole parts and a dotted line in gills. The lowest mRNA levels were generally observed in September in both tissue extracts. It should be noted that a secondary significant increase was observed in whole soft tissues in summer in St-Vaast but this was not confirmed in the other three sampling locations, on a quarterly basis. Though individuals were pooled, the data exhibited strong inter-individual variability, especially in winter.

3.4 Comparison between locations

We compared four populations located along the French coast at four periods of the seasonal cycle. The mRNA levels of p53 were measured in whole soft parts (Figure 5A) and gills (Figure 5B) of oyster from four sampling sites (Carnac, Cancale, Asnelles, St-Vaast, see

Figure 1) in March, June, September 2004 and January, March, June 2005. First, the t-test was performed at each quarter between the corresponding monthly-sampled location St-Vaast and the other quarterly-sampled locations to test whether the p53 mRNA level previously described in St-Vaast significantly differs in other locations. In gills and whole soft parts from animals collected in Carnac, Cancale and Asnelles, the mRNA levels were most often similar to those measured in the oysters from St-Vaast. Little significant difference (either increase or decrease) was observed between St-Vaast and the three other locations ($p<0.05$, t-test, Figure 5, symbol *) but obviously no clear relationship between the p53 expression level and the sampling location could be asserted from our data. Then, an ANOVA was performed between all months to study whether the mRNA level was homogeneously expressed throughout the annual cycle. This test revealed that the mean of the six sampling months could not be considered as homogeneous ($p<0.05$ for whole soft parts homogenate and $p<0.01$ for gills). The post-hoc multiple comparisons Student-Newman-Keuls test demonstrated that observed significant differences between months pointed to significantly higher values during winter months (March 2004, January 2005 or March 2005) in the four locations compared to summer months values (June 2004, September 2004 or June 2005). This was particularly clear in gills (Figure 5) and this may be smoothed in whole soft tissue mixtures, rather than individual tissues. In summary, the comparison between St-Vaast and the three other locations revealed that the seasonal pattern previously described in St-Vaast was confirmed in Cancale, Asnelles and Carnac with the current quarterly sampling frequency.

4. DISCUSSION

4.1 cDNA cloning of p53 in Crassostrea gigas

The present work reports the deduced amino acid sequences from the p53 cDNA cloned from the gills of the pacific oyster. This sequence showed a high homology with members of the p53-family from other molluscs. Despite the fact that the homology with human p53 is fairly low when considering the whole protein sequence (29% homology), this value is in accordance with the percentages usually observed between molluscs and human p53 (Muttray *et al.*, 2005). The Cg-p53 protein displays very strong homology in several conserved signature regions: 1 - the N-terminal transactivation domain, which provides the generic transactivation function and the binding site for MDM2, one of the main regulator of p53

stability (Momand et al., 1992; 1997). 2 - the proline-rich region, which was previously identified for complex roles as a protein-binding site and a specific regulator of apoptosis (Courtois et al., 2004). Unlike in oyster, human p53 contains three proline-rich region and additional proline residues. This proline-rich region may bind SH3-containing kinases involved in signal transduction (Walker & Levine, 1996; Venot et al., 1998). Two PxxP motifs have already been noticed in mussels (*M. edulis* and *M. trossulus*) and *Mya arenaria* homologues. 3 - the central region, which is the best conserved one with the presence of four sequence-specific DNA binding sites. 4 - the C-terminus, which includes several domains involved in oligomerization and regulation of the specific DNA-binding activity, as well as a non-specific DNA binding activity thought to be involved in non-specific p53-mediated DNA repair and in DNA/RNA reannealing (Wolkowicz & Rotter, 1997). In mammals, p53 functions as a tetrameric protein. In the C-terminus, the presence of conserved tetramerization domain, nuclear export signal, as well as conserved nuclear localization signal sequences suggests that oyster p53 homologue might be at least partially regulated through its oligomerization and subcellular localization like in mammals (Gottifredi & Prives, 2001). Muttray et al. (2005) suggested that, in p53 from mussel species (*Mytilus edulis* and *M. trossulus*), the nuclear localization signal has putatively a tripartite structure (NLS I, II and III) which is similar to human p53 (Shaulsky et al., 1990), although Kelley et al. (2001) indicated that *Mya arenaria* p53 may only have two NLS. In the deduced p53 amino acid sequence from *Crassostrea gigas*, two lysine-rich regions are clearly distinguishable in the C terminus, respectively homologous to NLSI and NLSIII, identified in human p53 (Shaulsky et al., 1990). The NLSII site of human p53 seems not present in the oyster homologue. However, it is highly speculative to conclude about the structure (bipartite or tripartite) of the NLS site in *C. gigas* p53.

The most divergent region in p53 is located between the transactivation domain and DNA-binding domain II. Indeed, the molluscs p53 homologues have a region which is 32 amino acid longer than in other organisms (including human) just before the proline-rich region. This is consistent with previous observation (Muttray et al., 2005) but the function of this longer region is not known.

4.2 p53 family isoforms: p53 or p63/p73?

The cloning and sequencing procedure led to the identification of one cDNA encoding for a p53 homologous protein but it must be pointed out that some other isoforms may exist in *Crassostrea gigas.* In man, two proteins presenting high structure homology with p53 have been discovered at the end of the 90's (Kaghad et al., 1997), namely p63 and p73 (reviewed in Irwin & Kaelin, 2001 and Murray-Zmijewski et al., 2006). The proteins p53, p63, p73 are encoded by two distinct genes: p53 and p63/p73. The p53 gene expresses one transcript (Soussi & May, 1996). It was shown that alternate promoters and splicing of p63/p73 gene leads to different isoforms (Yang et al., 2002): TA- and delta N- p63/p73. In man, these isoforms where shown to have overlapping and distinct functions (Nakagawa et al., 2002; Levrero et al., 2000). Moreover, human p53 and p73 sequences are not identical where they overlap, so that human p53 is more similar to mouse p53 than to human p73. In molluscs, several authors highlighted the putative existence of p53, p63 and p73 isoforms (Jessen-Eller et al., 2002; Stephens et al., 2001; Kelley at al, 2001; Cox et al., 2003; St-Jean et al., 2005; Muttray et al., 2007). Surprisingly, the data thus far (*Mya sp.* and *Mytilus sp.*) show that the central DNA binding domain is 100 percent conserved between all members of the p53 family within one species. This point diverges from higher vertebrates reports and arises the question about the number of p53 family genes in molluscs. To our knowledge, no genomic DNA sequence is available yet, neither for p53 nor for p63/p73 in molluscs, and this issue is still open.

In this study, the cDNA cloning was performed using degenerate oligonucleotides located in the central DNA-binding domain, usually well-conserved in all members of the p53 family, including p63 and p73. Due to the localization of these oligonucleotides, the presence or absence of the sterile alpha motif in the C-terminus of the full-length protein is a determinant point to discriminate between p53 and p63/p73 isoforms. Finally, together the homology of the newly identified sequence with other p53 members identified in other species, the absence of the SAM domain in the C-terminal region of the protein and the presence of a typical TGA stop codon in the cDNA sequence where it should occur for a protein of the predicted length of p53, support the idea that the cDNA cloned in *Crassostrea gigas* encodes a p53 protein. However, additional studies would be required to look for other hypothetical members of the p53 family in oyster and to further conclude the function of the protein encoded by the mRNA identified. If other p53 isoforms were to be isolated in the future in oyster as they have been in clam and mussel, the primers used for real time PCR in the present work would quantify the

expression of all isoforms if the central DNA binding domain were 100 percent conserved between all members of the p53 family in *C. gigas*.

Goodson et al. (2006) already underlined that the current nomenclature applied to invertebrates p53 family sequences may be misleading. According to Goodson et al (2006) and to the nomenclature used in higher vertebrates, our mRNA sequence could as well be considered as a p53 or a p63 lacking the SAM domain.

4.3 Distribution of p53 mRNA among tissues

This is the first report of p53 mRNA distribution among tissues in a molluscan species using the quantitative real time PCR technique. At the mRNA level, Van Beneden et al. (1997) examined the expression of a p53 homologue in three tissues of the soft shell clam *Mya arenaria* using Northern blot and RNAse protection assay. They showed that p53 mRNA was ubiquitously expressed in the three investigated tissues: adductor muscle, gills and gonad but using these semi-quantitative techniques, it remained difficult to assess the relative distribution among tissues. At the protein level, p53 was also found ubiquitously expressed among tissues in *Mya arenaria* (Kelley et al., 2001) and *Lamellides corrianus* (Mohanty, 2006). Our results are consistent with these observations.

Interestingly, Van Beneden et al. (1997) detected gender specific differences in gonads, with higher p53 mRNA levels in ovaries than in testis. However, they detected no significant differences in p53 mRNA level on an overall seasonal level in male or female gonad. The strong variability that we noticed in oyster whole soft tissues could be partly explained by such gender-specific differences in p53 expression in gonads.

4.4 Seasonal fluctuations of mRNA level

The regulation of the functions of p53 is controlled through several mechanisms including p53 transcription and translation, protein stability and post-translational modification (Lane, 1992), as well as p53 translocation between the cell nucleus and the cytoplasm (O'Brate and Giannakakou, 2003).

To our knowledge, the regulation of p53 at the mRNA level was much less described in vertebrates, than the regulation of the protein itself. In molluscs, reports of mRNA fluctuation are also very rare. Jessen-Eller et al. (2002) showed that p53 mRNA increases during embryonic development in the surf clam *Spisula solidissima*. Interestingly, they also showed that there is an inverse relationship between mRNA levels and protein levels of a p120 (a protein now believed to be a post-translationally modified p63/73 homologue) during embryonic development of the surf clam (Jessen-Eller et al., 2002). In a recent paper, Canesi et al. (2007) showed that 17-beta estradiol significantly decreased the transcription of the mussel p53 gene (about −50% with respect to control). Both studies showed that p53 mRNA level is potentially modified by endogenous (development) or exogenous (pollutants) factors. In the present paper, we show that mRNA level of p53 fluctuates strongly and in a season-dependent manner in the oyster *Crassostrea gigas*. Indeed, the p53 mRNA level is found naturally four-fold higher in winter than in summer in oyster tissues. It should be noted that we observed a secondary significant increase in whole soft tissues in summer in St-Vaast. This secondary summer increase was not detected in the three other locations with the current quarterly sampling frequency.

The data exhibited strong inter-individual variability, especially in winter, despite the fact that individuals were pooled. One factor that could, at least partially, explain this variability is oyster sex, which is known to influence many biological responses in mollusc. Indeed, the variability tends to be high when expression levels are high. Moreover, mixing all body tissue may smooth bigger differences existing in specific tissues. But the differences in expression levels between tissues were about two-fold whilst seasonal changes in gills were about four-fold. The fact that parallel seasonal changes were observed in gills and whole tissue extracts suggested that other tissues than gills also display a seasonal pattern.

The following arguments suggest that p53 mRNA level fluctuated in phase with season: 1- Winter mRNA level peaks coincided with minimal seawater winter temperature repeatedly over the studied period. 2- This main summer/winter trend was also observed in all four studied sampling locations on a quarterly basis. 3- p53 mRNA level displayed parallel patterns in gills and in whole soft tissues. Nevertheless, mRNA level of p53 may be influenced by many different processes, which may be in phase with seasonal changes. For example, the annual metabolic cycle of oysters is largely driven by reproduction and linked with temperature. The winter increase in p53 mRNA level coincided with the stage when oocytes grow in diameter (Lango-Reynoso et al., 2000) and the reserve tissue increases (Heude-Berthelin, 2000). Moreover, we observed that p53 mRNA level is lower in summer,

the end of the spawning period. Finally, the secondary significant increase in p53 mRNA observed in whole soft tissues in summer in St-Vaast, on a monthly basis, suggested that temperature is not the only driving parameter of the seasonal expression pattern.

The mRNA levels reflect a balance between their transcription and their degradation in the cells. The seasonal pattern observed for p53 transcripts has also been observed in the pacific oyster for other genes associated with cell stress (Farcy et al., 2007). Using different reference genes to normalize mRNA data confirmed the annual expression profile. Those cyclic fluctuations can be gene specific and reflect a response to seasonal environmental exposure of oysters to external stimuli, in which case the triggering parameters and the relationships with specific genes remain to be identified. Alternatively, the cyclic annual pattern may reflect the overall tissue mitotic activity, in relation with the degree of ripeness of the gonad because gonads in molluscs are often built up at the expense of other tissues with respect to growth and this is known to be influenced by season. Though this question is still unanswered, this natural seasonal pattern is crucial to bear in mind when using mRNA levels as molecular markers in field and laboratory experiments.

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Figure 1: Map showing the sampling locations along the French coast.

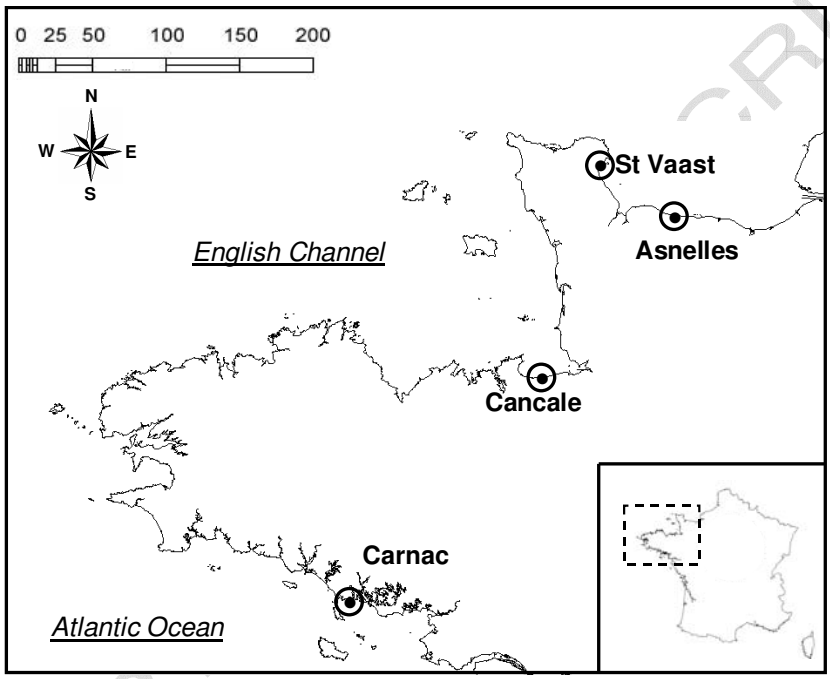


Figure 2: Protein sequence alignments (ClustalW v1.83) between *Crassostrea gigas* p53 protein (Genbank no. CAJ85664), human p53 (Genbank no. NP_000537), *Mytilus* spp. p53 (Genbank no. AAT72301 and AAT72302) and *Mya arenaria* p53 (Genbank no. AAF67733). Residues in black and grey highlight 100% identity and conserved substitution, respectively. The sequences used to design degenerate oligonucleotides deg_p53_F and deg_p53_R are outlined in blue below the human sequence. The location of p53_race1 and of the real-time PCR primer p53-QS and p53-QAS is outlined in green. The characteristic domains of the p53 family are highlighted in red above the mussel sequence. TAD: Transcriptional activation domain (or DNA binding domain I); ▲: conserved hydrophobic residues involved in MDM2 interaction in human; DBD: DNA binding domain; PxxP: Proline rich domain; TD: Tetramerization domain. Nuclear export signal (NES) and Nuclear localization domain (NLS) are outlined in blue below the C-terminus of the human sequence.

```
                                          TAD
                                  ----------
p53_Mytilus_tros      ------MSQASVSTTCTPSGPPMSQETFEYLWNTLGEVTQEGGYTNITSKESIDYAFSEA
p53_Mytilus_edu       ------MSQASVSTTCTPSGPPMSQETFEYLWNTLGEVTQEGGYTNITSKESIDYAFSEA
p53_Mya_are           MSHEALHKMSQVAIHGTLPNQPMSQETFEYLWHTLEEVTDNVDYTHINTRELDYSYD-DS
p53_Crassostrea_gig   -------MSQTLSPSSSVTGPPLSQEAFDYLWHSLDACTDHGNYTHIQQRDLDYSFENEN
p53_Homo_sap          ---------MEEPQSDPSVEPPLSQETFSDLWKLLPENN--------------------
                                           ▲   ▲   ▲

p53_Mytilus_tros      EDETSISVEKYRITSN-DSISDLLNPIIGQTTSASSMSPDSQTNIIGSSASSPYNDTITS
p53_Mytilus_edu       EDETSISVEKYRITSN-DSISDLLNPIIGQTTTASSMSPDSQTNIIGSSASSPYNDTITS
p53_Mya_are           EDGTSLQVEKFRINQHHTDVSDLLNPIIGTTSSSSMSPDSQTNISGSTASSPYQEMALTS
p53_Crassostrea_gig   EETTSLQIERFQIKSQQDSISDLLNPIIPQSTASSMSPDSQTGIIGSSTASSPYNDTITS
p53_Homo_sap          -----------VLSPLPSQAMDDLMLSPDDIEQWFTEDPGPDEAPRMPEAAPRVAPAPAA

                        PxxP        P-xxP                          DBD II
                                                  ----------------------
p53_Mytilus_tros      PPPYSPHTSMQSPIP-SVPSNTDYPGDYGFTISFSQPSKETKSTTWTYSESLKKLYVRMA
p53_Mytilus_edu       PPPYSPHTSMQSPIP-SVPSNTDYPGDYGFTISFSQPSKETKSTTWTYSESLKKLYVRMA
p53_Mya_are           PPPYSPHTNLTSPIP-TVPSNTNYPGDYGFEISFATPSKETKSTTWTYSDILKKLYVRMA
p53_Crassostrea_gig   PPPYSPHTSMTSPIP-TVPSNTDYAGDYGFQISFSQPSKETKSTTWTYSESLKKLYVRMA
p53_Homo_sap          PTPAAPAPAPSWPLSSSVPSQKTYQGSYGFRLGFLHSG-TAKSVTCTYSPALNKMFCQLA

                                                DBD III              p53-QS
                      ----                  --------------         ----->
p53_Mytilus_tros      TTCPIRFKCLRQPPQGCVIRAMPIFMKPEHVQEPVKRCPNHATSKEHNENHPAPTHLCRC
p53_Mytilus_edu       TTCPIRFKCLRQPPQGCVIRAMPIFMKPEHVQEPVKRCPNHATSKEHNENHPAPTHLCRC
p53_Mya_are           TTCPVRFKTLRQPPPGCVIRSMPIFMKPEHVQEAVKRCPNHATSKEFNENHPAPNHLVRC
p53_Crassostrea_gig   TTCPVRFKSQRQPPAGCIIRAMPIFMKPEHVQEPVKRCPNHATSKENNENHPAPTHLVRC
p53_Homo_sap          KTCPVQLWVDSTPPPGTRVRAMAIYKQSQHMTEVVRRCPHHERCSDSDGLAPPQHLIRVE
                                      ----->                          <--------
                                    deg_p53_F                         p53_race1
                                  p53-QAS                      DBD IV
                                    <----                 ------------------------
p53_Mytilus_tros      EHKLAKFVEDPYTSRQSVLIPHEIPQAGSEWVTNLFQFMCLGSCVGGPNRRPIQIVLTLE
p53_Mytilus_edu       EHKLAKFVEDPYTSRQSVLIPHEIPQAGSEWVTNLFQFMCLGSCVGGPNRRPIQIVLTLE
p53_Mya_are           EHKVSKYVEDPYTNRQSVLIPQETPQAGSEWVTNLFQFMCLGSCVGGPNRRPLQIVFTLE
p53_Crassostrea_gig   EHKLAKYTEDSYTSRQSVIIPHEQPQAGSEWVTNLFQFMCLGSCVGGPNRRPIQIVFTLE
p53_Homo_sap          GNLRVEYLDDRNTFRHSVVVPYEPPEVGSDCTTIHYNYMCNSSCMGGMNRRPILTIITLE

                                DBD V
                          ------------------
p53_Mytilus_tros      KDN-QVLGRRAVEVRICACPGRDRKADEKAALPPCKQS--PKKG-QKVNIINEITTVTPG
p53_Mytilus_edu       KDN-QVLGRRAVEVRICACPGRDRKADEKAALPPCKQS--PKKG-QKVNIINEITTVTPG
p53_Mya_are           KDN-QVLGRRCVEVRICACPGRDRKADERGSLPPMVSGGVKKSQMPKFSMGTEITTVSSG
p53_Crassostrea_gig   RDN-QVLGRRAVEVRICACPGRDRKADEKAALPPSKQS--PKKIGSKVSYGTEITTIVPG
p53_Homo_sap          DSSGNLLGRNSFEVRVCACPGRDRRTEEENLRKKGEPHHELPPGSTKRALPNNTSSSPQP
                                  <----
                                deg_p53_R                                    …/…
```

```
                                                TD
                                    ----------------------------------
p53_Mytilus_tros        GKKRKAEDEP-FTLSVRGRENYEILCRLRDSLELSSMVPQNQIDVYKQKQLDTNRQWLSM
p53_Mytilus_edu         GKKRKAEDEP-FTLSVRGRENYEILCRLRDSLELSSMVPQNQIDVYKQKQLDTNRQWLSM
p53_Mya_are             KKRKFEDDEQTFTLTVRGRENYDMLCKIRDSLEIAALLPQNQLQSLKQKQVEVQRQWLTN
p53_Crassostrea_gig     KKRKLDDDEDSYTLPVRGKENFEILCKLRDSLELSSMVPQNQVDRYKQQQVEVNRQWLSV
p53_Homo_sap            KKK--PLDGEYFTLQIRGRERFEMFRELNEALELKDAQAGKEPGGSRAHSSHLKSKKG--
                        -------                    -----------
                         NLS I                        NES

p53_Mytilus_tros        ILARENKNKLMKKVKRPQHRPGIKSRT
p53_Mytilus_edu         ILARENKNKLMKKVKRPQHRPGIKSRT
p53_Mya_are             VLAKEGKSRLIKKKHRPGKIIRHPLK-
p53_Crassostrea_gig     VLARESKNKLMKRKTKPGKVIKRPA--
p53_Homo_sap            -QSTSRHKKLMFKTEGPDSD-------
                             ------
                             NLS III
```

Figure 3: Distribution of p53 mRNA in tissues of *Crassostrea gigas* collected in St Vaast in February 2005: digestive gland (Dig. Gland), labial palps (Lab. Palps), posterior adductor muscle (Ad. Muscle), edge of the mantle (Edg. Mantle), gills and hemolymph. Values are given as mean and standard deviation, n=6 individuals, except for hemolymph where the values correspond to n=2 pools of 3 individuals. Different letters indicate significant differences between means of values (Newman Keuls test; p<0.05).

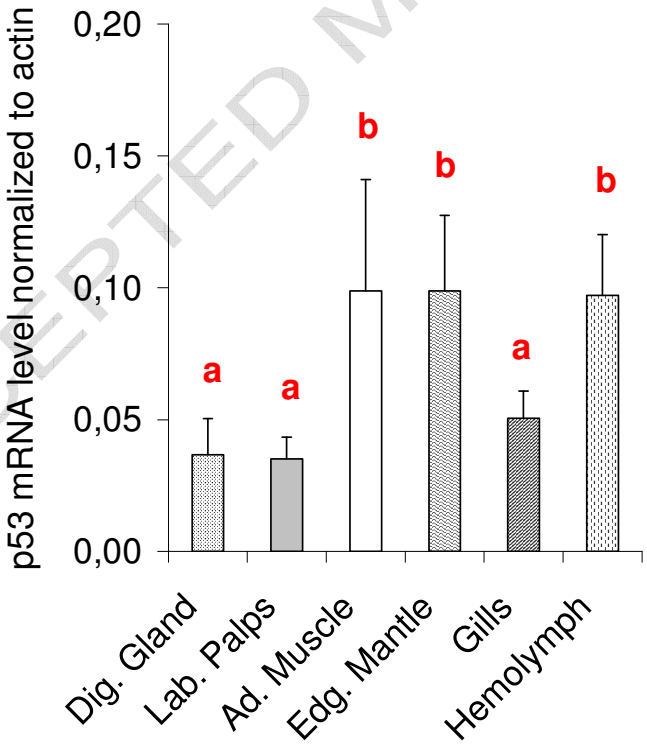


Figure 4: Mean and standard deviation values for p53 mRNA levels in gills (dotted line) and whole soft tissues (solid line) in oysters collected monthly in St-Vaast between January 2004 and September 2005. Vertical dotted lines and solid lines correspond to *sd* values in gills and whole soft tissues, respectively. Symbol * means a statistically highly significant difference (p<0.001) between the two paired data (t-test on log-transformed data, n=4 pools of 5 individuals) and the associated value corresponds to their ratio. The blue bands indicate the periods displaying seawater temperature below 10 °C.**

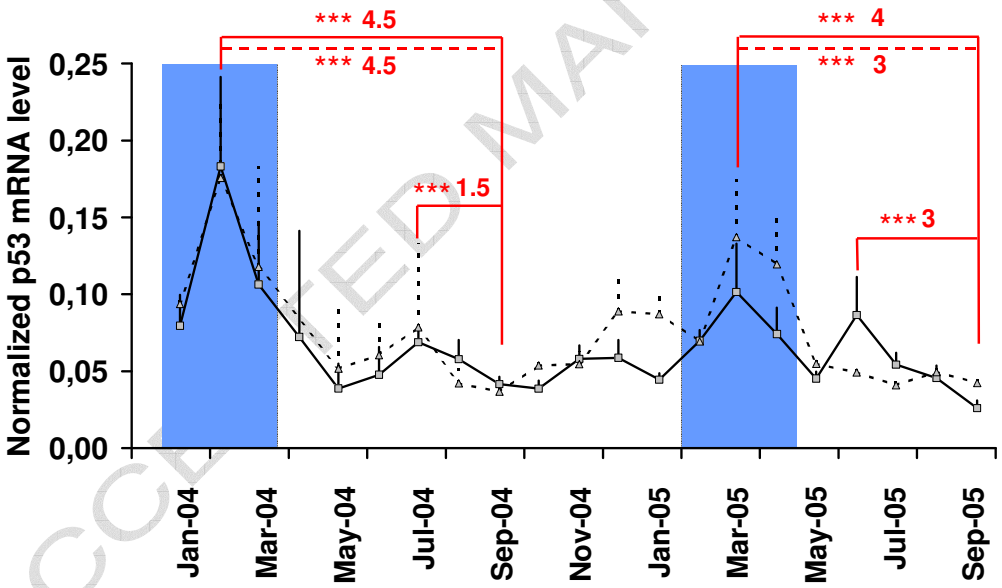
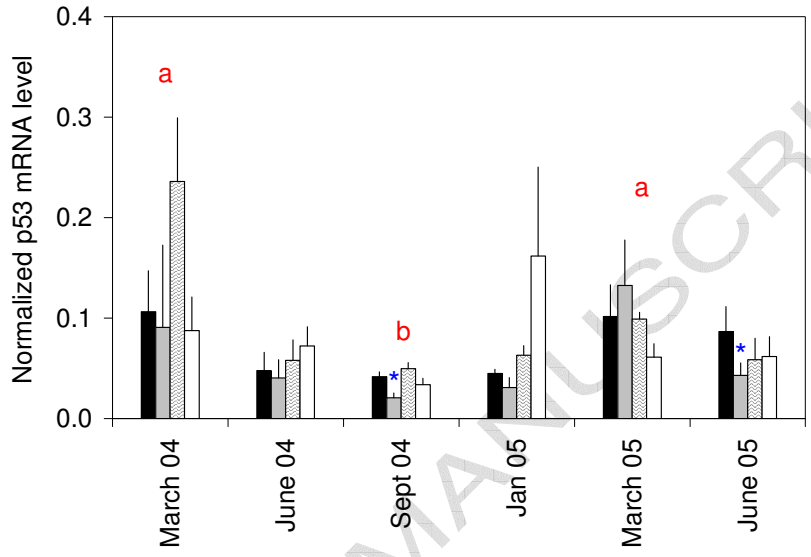


Figure 5: p53 mRNA levels in whole soft parts (A) and gills (B) from oysters collected quarterly in St-Vaast (black), Asnelles (grey), Cancale (hatched) and Carnac (white). * symbol placed above a bar for a quarterly-sampled location (Asnelles, Cancale, Carnac) means a statistically significant difference in mRNA level ($p<0.05$) between this location and the corresponding monthly-sampled site St-Vaast (t-test, n=4 pools of 5 individuals). Different letters indicate significant differences between means of values within the same sampling period (Newman Keuls test; $p<0.05$). Letter a and b respectively highlighted winter and summer season.

(A)



(B)

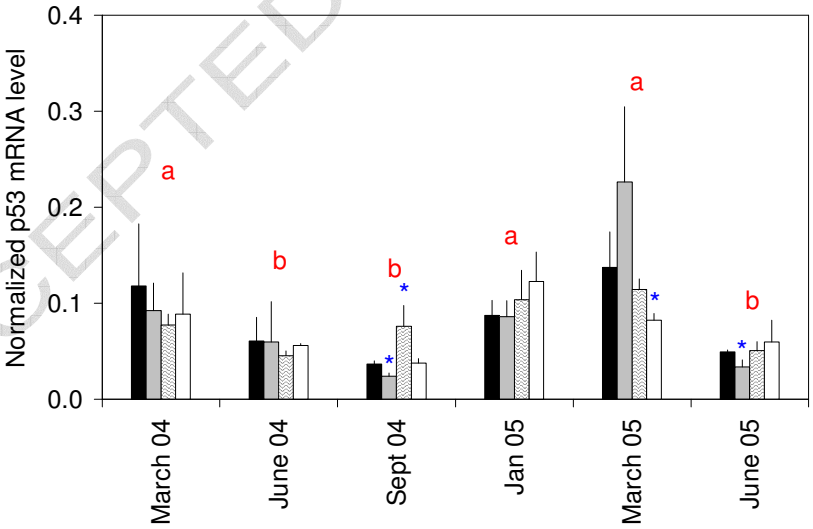


Table 1: Primers used for mRNA cloning and mRNA quantification by real time PCR.

Primer use	Forward primer	Reverse primer
Search for a specific fragment using degenerate oligonucleotides	deg_p53_F 5′-ATG CCN ATH TWY ATG AA-3’	deg_p53_R 5′-GGR CAN GCR CAD AT-3′
5’ RACE PCR	Generacer™ 5’ primer 5’ GCACGAGGACACTGACATGGACTGA 3’	p53 race1 5’ GCACCTCACCAAATGAGTCGGAGC 3’
3’ RACE PCR	p53_QS 5’ ACCCAGCTCCGACTCATTT 3’	Generacer™ 3’ primer 5’ GCTGTCAACGATACGCTACGTAACG 3’
Real time PCR p53	p53_QS 5’ ACCCAGCTCCGACTCATTT 3’	p53_QAS 5’ TCATGGGGGATGATGACAC 3’
Real time PCR actin	actin_F 5’ GCCCTGGACTTCGAACAA 3’	actin_R 5’ CGTTGCCAATGGTGATGA 3’
Real time PCR GAPDH	GAPDH_F 5’ TTGTCTTGCCCCTCTTGC 3’	GAPDH_R 5’ CGCCAATCCTTGTTGCTT 3’
Real time PCR 18S	18S_F 5’ CGGGGAGGTAGTGACGAA 3’	18S_R 5’ ACCAGACTTGCCCTCCAA 3’

Table 2: Homology (%) between the amino acid sequence of p53 protein from *Crasssostrea gigas* (Genbank no. CAJ85664) and other p53 proteins from *Mya arenaria*, *Mytilus edulis*, *Mytilus trossulus* and *Homo sapiens* (ClustalW 1.83).

p53 homologues	Genbank accession number	Homology with *Crassostrea gigas* p53 (CAJ85664)
Mya arenaria p53	AAF67733	67 %
Mytilus edulis p53	AAT72301	71 %
Mytilus trossulus p53	AAT72302	71 %
Homo sapiens p53	NP_000537	29 %