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Seasonal changes in mRNA encoding for cell stress markers in the oyster *Crassostrea gigas* exposed to radioactive discharges in their natural environment

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Abstract:

The North Cotentin area (Normandy, France) hosts several nuclear facilities among which the AREVA reprocessing plant of La Hague is responsible for controlled discharges of liquid radioactive wastes into the marine environment. The resulting increase in radioactivity is very small compared to natural radioactivity. However, concerns about environment protection prompted the scientific community to focus on the effects of the chronic exposure to low concentrations of radionuclides in non-human biota. This study contributes to the evaluation of the possible impact of radioactive discharges on the oyster *Crassostrea gigas* in the field. Real-time polymerase chain reaction was used to quantify the expression levels of genes involved in cell stress in the oyster. They included members of the heat shock protein family (Hsp70, Hsc72, Hsp90), superoxide dismutase (SOD) and metallothionein (MT). Times series measurements were built from periodic samplings in the natural environment in order to characterize the natural variability as well as possible seasonal fluctuations. The genes studied exhibited a general seasonal expression pattern with a peak value in winter. The data inversely correlated with seawater temperature and the nature of the relationship between gene expression and temperature is discussed. In parallel, oysters were collected in four locations on the French shores, exposed or not to radioactive liquid wastes from the nuclear facilities hosted in the North Cotentin. The comparison of data obtained in the reference location on the Atlantic coast (not exposed) and data from oysters of the English Channel (exposed) gave no evidence for any statistical difference. However, because of the complexity of the natural environment, we cannot rule out the possibility that other parameters may have masked the impact of radioactive discharges. This dense set of data is a basis for the use of the expression levels of those genes as biomarkers to address the question of the possible effects of chronic exposure of the oyster to low concentrations of radionuclides in controlled laboratory experimental conditions.

Keywords: Radioactive discharges; Seasonal variations; mRNA; Heat shock protein; Metallothionein; Superoxide dismutase; *Crassostrea gigas*

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ABSTRACT

The North Cotentin area (Normandy, France) hosts several nuclear facilities among which the AREVA reprocessing plant of La Hague is responsible for controlled discharges of liquid radioactive wastes into the marine environment. The resulting increase in radioactivity is very small compared to natural radioactivity. However, concerns about environment protection prompted the scientific community to focus on the effects of the chronic exposure to low concentrations of radionuclides in non-human biota. This study contributes to the evaluation of the possible impact of radioactive discharges on the oyster Crassostrea gigas in the field. Real-time polymerase chain reaction was used to quantify the expression levels of genes involved in cell stress in the oyster. They included members of the heat shock protein family (Hsp70, Hsc72, Hsp90), superoxide dismutase (SOD) and metallothionein (MT). Times series measurements were built from periodic samplings in the natural environment in order to characterize the natural variability as well as possible seasonal fluctuations. The genes studied exhibited a general seasonal expression pattern with a peak value in winter. The data inversely correlated with seawater temperature and the nature of the relationship between gene expression and temperature is discussed. In

parallel, oysters were collected in four locations on the French shores, exposed or not to radioactive liquid wastes from the nuclear facilities hosted in the North-Cotentin. The comparison of data obtained in the reference location on the Atlantic coast (not exposed) and data from oysters of the English Channel (exposed) gave no evidence for any statistical difference. However, because of the complexity of the natural environment, we cannot rule out the possibility that other parameters may have masked the impact of radioactive discharges. This dense set of data is a basis for the use of the expression levels of those genes as biomarkers to address the question of the possible effects of chronic exposure of the oyster to low concentrations of radionuclides in controlled laboratory experimental conditions.

1. INTRODUCTION

The North Cotentin area (Normandy, France) hosts several nuclear facilities, including the AREVA reprocessing plant in La Hague, which is responsible for controlled discharges of liquid radioactive waste into the marine environment. These discharges result in measurable amounts of a few artificial radionuclides in the vicinity of the input source. The resulting increase in radioactivity is small compared to natural radioactivity. However, oyster farms are located in this area and the oysters may be slightly labelled. Recently, concerns about environmental protection have prompted the scientific community to focus on the effects of ionizing radiation on non-human biota. This study contributes to the evaluation of the possible impact of radioactive discharges on the oyster Crassostrea gigas in the field. Since oyster physiology is known to be strongly influenced by the reproductive cycle, its response to a given stress is likely to fluctuate seasonally. In this context of chronic exposure, it was necessary to address the question of possible seasonal fluctuations in the oysters' responses.

We monitored seasonal changes in the expression of genes involved in the response to cell stress in different locations, which either were or were not exposed to artificial radionuclides released by the nuclear industry. We focussed on mRNA levels of particular genes, known, on the basis of radiobiology literature, to be involved in the response to

ionizing radiation. The genes dealt with in this study are involved in the chaperone protein function: the heat shock protein family (Hsp70, Hsc72, Hsp90) (Ehrhart et al., 1988; Davidoff et al., 1992; Sierra-Rivera et al., 1993; Matsumoto et al., 1995; Kang et al., 2002; Calini et al., 2003); and in regulation of oxidative stress: superoxide dismutase (SOD), metallothionein (MT) (Greenstock et al., 1987; Yamaoka et al., 1994; Otero et al., 1996; Cai et al., 1999; Cai and Cherian, 2003; Guo et al., 2003). Real-time polymerase chain reaction (PCR) was used to quantify mRNA encoding for these markers in comparison with mRNA encoding for actin, as a reference gene. RNA was extracted from whole oyster soft part homogenates as well as from isolated gills. Samples were collected monthly and quarterly on the French Atlantic and Channel coasts in order to build time series measurements of radionuclide labelling and gene expression. Statistical analysis of these data was performed, first to characterize the ranges of fluctuations in these markers in the natural environment, as well as possible seasonal variations, and secondly, to compare populations that either were or were not exposed to radioactive discharges by the AREVA reprocessing plant.

2. METHODS

2-1 Sampling

Oysters were obtained from oyster farms located in the sampling sites. 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 just before sampling.

For radionuclide concentration measurements, oysters were purchased monthly in St-

Vaast (STV) from July 2002 to February 2005, and quarterly in Asnelles (ASN), Cancale (CCL) and Carnac (CRC) (Fig. 1) from March 2004 to January 2005. The whole of the soft parts was separated from the shell and dried at 90°C to a constant dry weight and then ground into fine powder. The dry material was conditioned in a suitable geometry for gamma spectrometry analysis. On a few samples only, another aliquot of dry material was calcined at 450°C and the ash was sent for americium, curium and plutonium radiochemistry and alpha spectrometry analysis by the Environmental Radioactivity Measurement Laboratory (IRSN, Orsay, France).

For gene expression experiments, oysters were collected monthly in St-Vaast and quarterly in Asnelles, Cancale and Carnac between January 2004 and September 2005. At these four locations, the gills of 20 oysters and all the soft parts of 20 other oysters were immediately dissected, on the spot after sampling. After dissection, the samples were immediately frozen in liquid nitrogen. Back at the laboratory, the oyster extracts were crushed in a ball crusher, in buckets cooled in liquid nitrogen. After this step, five individual oysters were pooled together as one.

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 discard genomic DNA contamination, total RNA was digested with DNAse I Amplification Grade (Sigma-Aldrich) and an aliquot of DNAse-treated RNA was then subjected to a PCR with actin primers to check the absence of genomic DNA.

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 (Table 1). Their efficiency was tested using the standard curve method. A dilution range was performed for each pair of primers to obtain a standard curve by plotting the cycle threshold as a function of the log of the total amount of starting RNA (reverse-transcripted). Primer pairs showing a good efficiency level (100 ± 5%) on a range of cDNA concentrations of four orders of magnitude were kept for quantification.

Real-time PCR was performed in a MiQ Cycler (Biorad). All determinations were carried out in duplicate. 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 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 melting curve was used to check whether the amplification products had the expected Tm.

2-4 Normalization of mRNA

A preliminary task was to specifically address the choice of the reference gene to quantitatively express gene expression. To analyse gene expression level, mRNA quantities were normalized to actin mRNA, a cytoskeleton protein, GAPDH mRNA, a gene involved in glycolysis often used as reference, and 18S ribosomal RNA, which accounted for total RNA, in order to check the influence of the reference gene. Seasonal expression patterns were found to be very similar when normalized to these three classically used reference RNAs (data not shown) and actin mRNA was finally used for all subsequent normalization.

2-5 Seawater temperature measurements

The English Channel is characterized by exceptionally strong tidal currents and low depth, so the water column is considered homogenous. Temperature was measured up-tide from the shore monthly with a digital thermometer (precision: 0.1°C). The measurements were performed in Goury, near the St-Vaast sampling site.

2-6 Statistical analysis

Statistical analysis was carried out using Statgraphics Centurion XV software (StatPoint, Inc., http://www.statgraphics.com/). Values were expressed as means ± SD of four pooled oyster extracts from 5 individuals. Statistically significant differences between two samples were determined using a Mann-Whitney non-parametric rank test; * $p \leq 0.05$ was accepted as significant. Linear regression analysis was performed to estimate correlations (r) between water temperature versus mRNA fluctuations.

A principal component analysis was carried out, using SPAD 6.0 software (SPAD

TEST&GO, http://www.testandgo.com/), to characterize the main trends of the data set with respect to all descriptors: gene, month, type of extract and location.

3. RESULTS

3-1 Radionuclide concentrations

Gamma spectrometry analysis of all four locations of oyster samples (whole soft parts) showed the presence of natural ^{40}K every month, in the range 316-452 $Bq.kg^{-1}$ dry weight (dw). In CCL, ASN (remote from AREVA reprocessing plant liquid waste input) and CRC (Atlantic coast, unexposed to discharges from AREVA plant), no artificial radionuclides were detected by gamma spectrometry. In STV, ^{60}Co was detected 9 times and ^{137}Cs was detected 3 times, out of 32 monthly samplings between Jul-02 and Feb-05, at concentrations generally less than 0.2 $Bq.kg^{-1}dw$ and 0.5 $Bq.kg^{-1}dw$, respectively (Table 2). Months when no artificial radionuclides were detected by gamma spectrometry are not shown in Table 2.

Radiochemistry of transuranic elements and alpha spectrometry analysis was performed on one sample from CRC (Atlantic coast), one sample from CCL and three samples from STV. The results expressed in $Bq.kg^{-1}dw$ are presented in table 3 and though there were very few data, it can be noted that, compared to the background level in CRC (Atlantic coast), a slight labelling could be detected in the Cotentin locations.

3-2 Water temperature

Seawater temperature was measured in the North Cotentin area, close to the AREVA reprocessing plant. Maximum water temperatures were reached during the summer period (August-September) with values around 18°C, while minimum values of 7°C were observed in February. These cold periods (below 10°C, grey bands) are outlined in figures 2 and 3.

3-3 Seasonal variations of stress-inducible gene expression

Monthly recorded values of mRNA levels in gills and whole soft parts in oysters from STV between January 2004 and September 2005 are shown in figure 3. Genes involved in protein refolding are Hsp70 (Fig. 3a), Hsc72 (Fig. 3b) and Hsp90 (Fig. 3c), and those involved in oxidative stress regulation are SOD (Fig. 3d) and MT (Fig. 3e).

For all five stress-inducible genes, animals collected monthly in STV showed seasonal fluctuations in mRNA levels in both isolated gills and whole soft parts. Firstly, it appears that the mRNA level peaked in the winter period: January-February-March 2004 and February-March-April 2005. For each gene studied and for both gills and soft part homogenate, the Mann-Whitney test was performed on pairs of maximal (winter, summer) and minimal (September) values. In figure 3, paired values were linked with a solid line in whole parts and a dotted line in gills.

Another critical period of the annual cycle was found in the summer between June and August, during which whole soft part values exhibited a significant increase compared to May and September mRNA levels. The lowest mRNA levels were generally observed in May and September in both gills and soft part homogenate. The ratio of this summer

mRNA level increase was generally smaller than the ratio of winter increase, except for SOD and Hsc72, which exhibited particularly strong increases in the summer. The latter genes showed a 4-fold significant increase ($p<0.05$) of mRNA level in June-July-August 2004 and June-July 2005 in the whole soft parts compared to May and September of the same year. This summer increase in mRNA level was not observed in gills.

3-4 Relationship with temperature

The above results showed that mRNA levels of stress-inducible genes apparently exhibited a temperature-related pattern. Indeed, the temperature hits its low-point below 10°C when mRNA levels were at their highest.

In order to study whether stress-induced genes' mRNA levels are related to temperature, a linear regression analysis between mRNA levels in STV and water temperature was performed and the correlation coefficients were calculated in both whole soft parts and gills (Table 4). The inverse correlation between seawater temperature and mRNA levels in STV was generally better in the gills than in the whole soft parts. This was expected to be due to the summer increase observed in whole soft parts and not in gills.

3-5 Comparison between locations

In order to compare populations that either are or are not exposed to radioactive discharges by the AREVA reprocessing plant, we studied the differences between CRC (reference site) and the other locations at four periods of the year: January, March, June and September. Values of mRNA levels of stress-inducible genes in whole soft parts of oyster from the four sampling sites (CRC, CCL, ASN, STV) are shown in figure 4 for January,

March, June, September 2004 and January, March, June 2005: Hsp70 (Fig. 4a), Hsc72 (Fig. 4b), Hsp90 (Fig. 4c), SOD (Fig. 4d) and MT (Fig. 4e). For each gene studied, a Mann-Whitney test was performed between the values for CRC and each of the other three sampling locations (CCL, STV and ASN).

In animals collected in CCL, ASN and STV, the whole soft parts mRNA levels were most often closely similar to those measured in the oysters from CRC. Data obtained on the same studied genes, in isolated gills from different oysters, sampled at the same time, in the four locations, were similar (data not shown). Only very few statistically significant differences could be found between the four locations. These significant differences indicated either higher or lower values in one of the three locations (CCL, ASN and STV), compared to the unexposed site of CRC. For the genes considered, the ratios between the lowest mRNA level in September and highest in January or March were similar in the four sites. The previously described seasonal pattern in STV was confirmed in CCL, ASN and CRC. However, the summer increase, previously described in the whole soft parts in STV, could not be observed in the three other locations with the current quarterly sampling frequency.

3-6 Principal Component Analysis

A Principal Component Analysis (PCA) was performed on the mean values of mRNA levels obtained in gills and whole soft parts for the five genes studied, the four sampling locations and the six months (March-04, June-04, September-04, January-05, March-05 and June-05).

The principal axes are constructed from the dispersion of the data for the quantitative parameters (gene expression). The correlation circle (Fig. 5) showed that all variables

were negatively correlated to the first axis (size effect). The first two components represented 79% of the total variance. It was interesting to examine the correlation of these components with the genes (Table 5). The correlation among descriptors is given by the angle between descriptor axes. The closer to unity (unsigned value), the stronger the correlation. This correlation revealed that all genes explained component 1 well.

The illustrative parameters (location and month) are positioned a posteriori according to the existing principal axis. For each axis and each illustrative parameter, the test value expresses the statistical significance of the parameter's coordinate on this axis. The matrix of the test values is given in table 6 for the illustrative parameters (location, month). We can see that the parameter "month" was well represented on axis 1 (absolute test value>2), whereas the parameter "location" was not (absolute test-value<2). This means that, unlike the locations, the sampling months were significantly linked to the first axis, except for March 2004. Finally, axis 1 clearly separated data as a function of time and this first axis showed a strong contrast between winter and summer (Fig. 6). In table 6, axis 2 also revealed an opposition between September 2004 (test-value=-2.9) and June 2005 (test-value=2.4). However, on this axis 2, the test values did not allow for interpretation of the "location" parameter.

4. DISCUSSION

The main goal of this study was to compare oysters from locations that either are or are not exposed to liquid radioactive waste discharges in the marine environment. The Cotentin area in France is a typical example of the potential impact of the nuclear industry on the natural environment because this is where the La Hague AREVA reprocessing plant is sited. Dispersion of radioactive liquid discharges from the Cotentin area into the English

Channel is well documented with respect to hydrodynamics and it has been shown to move towards the North Sea (see Bailly du Bois and Dumas, 2005 for a review). Among the four sampling locations, Carnac, located on the Atlantic coast, was thus used as the reference site. Concentrations of radionuclides in different biological compartments of the marine environment on the French Atlantic and the English Channel coasts between 1993 and 2003 were recently reviewed (Fiévet and Germain, 2004). Regarding gamma emitter radionuclides, we should point out that ^{40}K was found in oyster soft parts from all four sampling locations. This is a natural radionuclide, present at background level. Labelling by AREVA liquid radioactive waste was detectable in St-Vaast, which is downstream of the plant discharge dispersal, with the presence of ^{60}Co and ^{137}Cs. However, it should be recalled that part of the ^{137}Cs present in oysters in the Cotentin area is a result of atmospheric nuclear weapons tests which lasted up to the 70s (Fiévet and Germain, 2004). In any case, these artificial gamma emitter radionuclide concentrations are at least 3 orders of magnitude lower than natural ^{40}K. A few analyses of transuranic elements in oyster samples were performed in this work and the results showed that these alpha emitter radionuclides were detected on the Atlantic coast (Carnac). Their presence in the reference site is also a consequence of past atmospheric nuclear weapons tests. Labelling in oysters around the Cotentin Peninsula was clearly detected with up to 30-fold higher alpha emitter concentrations than in Carnac. The marine environment is also characterized by the presence of ^{210}Po, another element of natural origin (the result of natural ^{238}U decay). This natural alpha emitter radionuclide was analysed in oysters from the same sampling locations, and concentrations in soft parts were found in the 100-1000 $Bq.kg^{-1}$dw range (Connan, personnal communication). Here again, the ratio of several orders of magnitude between artificial and natural alpha emitter radionuclide concentrations should be emphasized. The fluctuations of radionuclide concentrations in seawater in the English

Channel are well documented. Many measurements and accurate models have been developed and fine-tuned to account for dispersion of liquid waste (Bailly du Bois and Dumas, 2005). At the distance from the source of input where St-Vaast is located, radionuclide concentrations are quite stable compared to the vicinity of the AREVA pipe where sharp changes reflect the kinetics of releases by the plant (Fiévet and Plet, 2003; Fiévet et al., in press). In Cancale and Asnelles, because of detection limits, present gamma spectrometry data provided no evidence for the labelling of oysters by radioactive discharges from the AREVA reprocessing plant. However, in past decades, radioecology studies in the English Channel, considering other marine indicators and/or other radionuclides, have shown that these two locations are in the area of influence of these discharges. From this brief overview of radioecology, the impact of the nuclear industry on oysters in the English Channel is expected to be fairly constant and probably low, especially considering natural radioactivity. Extremely sensitive tools are thus required to conduct research into any possible impact.

Most literature available on the effects of radiations on cellular functions is devoted to mammals, exposed to high and acute doses. Few studies have conducted research into the effects of chronic exposure of lower vertebrate and invertebrate species to low doses of ionizing radiation (Cooley, 1973; Baptist et al., 1975; Woodhead, 1977, Rackham and Whoodhead, 1984; Theodorakis and Shugart, 1998; Hinton, 2000; Mihok, 2004; Hagger et al., 2005; Jha et al., 2005) and none into mRNA. However, on the basis of radiobiological knowledge, one can focus on a number of cellular functions, expected to be involved in the response to ionizing radiation. Ionizing radiation results in radiolysis of water molecules, which produces reactive oxygen species (ROS), and more generally damage to potentially all molecules present, altering their functions. Cell responses to this damage

involve chaperone proteins (Ehrhart et al., 1988; Davidoff et al., 1992; Sierra-Rivera et al., 1993; Matsumoto et al., 1995; Kang et al., 2002; Calini et al., 2003) and defence against oxidative stress (Greenstock et al., 1987; Yamaoka et al., 1994; Otero et al., 1996; Cai et al., 1999; Cai and Cherian, 2003; Guo et al., 2003), among others. The recent development of the real-time PCR technique following reverse-transcription makes it possible to quantify messenger RNA precisely and thus determine the expression level of specific genes. One key issue of using the real-time PCR technique is the choice of the reference RNA (Thellin et al., 1999; Goidin et al., 2001; Kim et al., 2003; Andersen et al., 2004; Ropenga et al., 2004; Bustin et al., 2005; Huggett et al., 2005; Wong and Medrano, 2005), this being particularly relevant for in situ studies with periodic mRNA level recording. We verified that the observed variation pattern of stress-inducible genes was not due to the variation of actin itself.

A prerequisite for the use of mRNA levels of genes as markers of oyster stress was to figure out their natural range of fluctuation to serve as a basis for all subsequent comparisons and to be able to assess the significance of observed changes.

In the entire data set obtained in STV, we observed overall high levels of mRNA between January and April in both gills and soft part homogenate. In whole soft parts, a secondary increase in mRNA level was noticed in the summer period between June and August, especially for SOD and Hsc72 mRNA. Two distinct hypotheses could be proposed to explain these fluctuations: either they were seasonal or they reflected changes in the source of a local pollutant such as radionuclides released by the AREVA plant or another stressor, which may or may not have been anthropogenic. The hypothesis of changes in the impact of liquid radioactive waste from the AREVA plant seemed unlikely for the

following reasons: at that distance from the point of release, radionuclide concentrations in seawater are quite stable and radionuclide transfer kinetics between seawater and biological compartments smooth out changes in marine species even further (Fiévet and Plet, 2003; Fiévet et al., in press). Another argument came from the comparison between data obtained in St-Vaast and the three other sampling locations, including the reference site on the Atlantic coast, which provided no evidence for any statistically significant systematic difference in St-Vaast (further discussed below). As far as fluctuations of other possible pollutants are concerned, pressure by unidentified sources of environmental annual cyclic stressor obviously cannot be ruled out. Data from the seawater quality-monitoring program suggested that the four locations did not strongly differ regarding metals, polycyclic aromatic hydrocarbons (PAH) and polychlorinated biphenyl (PCB) (Ifremer, 2005a; Ifremer, 2005b; Ifremer, 2005c). The hypothesis of seasonal fluctuations is supported by the following arguments: synchronicity between winter mRNA level peaks and seawater winter temperature was observed repeatedly over the whole time period and the main seasonal trend was confirmed with quarterly samplings in all four locations studied.

On the other hand, a direct role of temperature on the mRNA level of stress-inducible genes cannot be asserted from the data obtained, even if 60% to 80% of the variance was explained using the hypothesis of an inverse linear relationship, depending on the gene and the oyster extract considered. Indeed, mRNA levels of stress-related genes may be influenced by physiological processes other than stress, which are in phase with seasonal temperature changes. For example, the annual metabolic cycle of oysters is largely driven by reproduction and linked to temperature. We observed that the period of increase in the mRNA level (January-February-March) coincided with gametogenesis, more precisely the stage in which the oocyte grows in diameter (Lango-Reynoso et al., 2000) and the reserve

tissue increases (Heude-Berthellin, 2000). Moreover, we observed that mRNA levels fell in August-September every year (2004, 2005): this was in phase with the end of the spawning period. This decrease after the spawning period was observed in Corbicularia, with minimum values of MT protein concentrations observed after the spawning period (Baudrimont et al., 1997).

Comparing the different locations was a major goal of this study. On the basis of quarterly samplings, seasonal fluctuations were observed in all four sites with similar magnitude. Mann-Whitney test comparisons between the reference site (Carnac, Atlantic coast) values and the English Channel sites values pointed out some scattered statistically significant differences. However, no clear tendency (increase or decrease) in mRNA levels could be associated with the exposure of sampling locations to liquid radioactive waste from the AREVA reprocessing plant in La Hague. We cannot exclude the possibility that differences could be hidden or counteracted by other parameters. However, in the end, our data turned out to appear strongly influenced by the season, even though we focused on artificial radionuclides in North Cotentin in this study. A principal component analysis was performed to look further into this extensive dataset (sampling locations, genes, sampling quarters). This multivariate analysis showed that the dataset was highly structured by seasonality, with a strong contrast between winter months (January, March) and summer months (June, September), for all four locations. Neither of the first two axes was generated by the "location" parameter. This meant that this descriptor did not strongly structure the dispersion of the data.

Since the genes considered in this study are presumably influenced by a variety of biotic and abiotic factors, one may wonder whether any seasonal patterns exist. Interestingly, the

genes studied showed the same general fluctuation pattern. Most data available from the literature on environmental ecotoxicological studies regarding those stress markers were obtained at the level of proteins or physiological functions. However, it is worth briefly comparing existing results with the actual pattern observed at transcriptional level.

Regarding oxidative stress, previous studies showed a correlation between antioxidant defences and seasonality, with respect to the reproductive period and food intake (Viarengo et al., 1991; Solé et al., 1995). In particular, in the summer, temperature and food availability induce oxygen consumption and cellular oxyradical generation, which are offset by increasing antioxidant defences. Viarengo et al. (1991) suggested that the decrease in activity of the antioxidant defence system during the winter could be directly responsible for an enhanced susceptibility of mussels to oxidative stress in this period. Similarly, Sheehan and Power (1999) emphasize that oxidative stress is a highly seasonal phenomenon in bivalves. In addition, the modulation of oxidative stress by environmental pollutants obviously interferes and makes interpretation of biomonitoring data difficult. The fluctuation pattern obtained for Crassostrea gigas SOD mRNA in this study corroborates the general consideration that the summer is a stressful period with a high level of oxidative stress. But we also underlined that the colder period between January and April was characterized by even higher SOD mRNA levels, which we cannot currently explain, on the basis of literature.

Metallothionein has been widely used as a biomarker in field and laboratory studies, in particular in relation to its function in metal detoxification (Baudrimont et al., 1997; Ringwood et al., 1998; Geffard et al., 2001, 2002, 2005; Tanguy et al., 2002; Irato et al., 2003; Bodin et al., 2004; for a review, see Amiard et al., 2006). MT was also clearly shown to be involved in scavenging free radicals during oxidative damage in vertebrates

(Sato and Bremner, 1993; Chubatsu and Meneghini, 1993; Dalton et al., 1994; Cai et al., 1999; You et al., 2002). In molluscs, the antioxidant role of metallothionein has been shown in bivalves and gastropods (Roesijadi et al., 1997; Anderson et al., 1999; Viarengo et al., 1999; Viarengo et al., 2000; Leung and Furness, 2001; Cavaletto et al., 2002; Bebianno et al., 2005), providing evidence that metallothionein is effective in protecting cells and the entire organism against oxidative stress. Amiard et al. (2006) discussed the confounding factors affecting concentrations of MT: temperature (Serafim et al., 2002) and salinity (Leung et al., 2002). Among confounding factors, the reproductive cycle was found to be strongly correlated with the annual variation in metallothionein level of the Asiatic clam (Corbicula fluminea) in an unpolluted site in France (Baudrimont et al., 1997). Interestingly, we observed that MT mRNA levels fell in August-September every year (2004, 2005) in phase with the end of the spawning period in the oyster.

Concerning the Hsp family gene, increased levels of Hsps have been associated with fluctuations in environmental temperatures in mussels and marine snails (Chapple et al., 1998; Hofmann and Somero, 1995; Tomanek and Somero, 1999, Minier et al., 2000; Hofmann et al., 2002) and enhanced thermal tolerance in oysters (Clegg et al., 1998, Piano et al., 2002). However, as underlined by Encomio and Chu (2005), it has not been unequivocally demonstrated that seasonal variation in Hsps corresponds directly to seasonal temperature changes. These authors showed in Crassostrea virginica, that total amounts of Hsp70 did not correlate positively with seasonal variations in temperature. Similarly, subtidal mussels (Mytilus sp.) were shown to express higher levels of Hsp70 in winter than in summer (Roberts et al., 1997). Other factors such as salinity or disease were reported to alter Hsp expression (Shumway and Koehn, 1982; Werner and Hinton, 2000). In our study, we observed a small increase in mRNA levels of Hsp family members

(Hsp70, Hsc72 and Hsp90) in whole soft tissue of oysters during the summer period but also high mRNA levels in the winter between January and April.

5-CONCLUSION

We did not find any evidence for any relationship between changes in these mRNA levels and oysters' exposure to liquid radioactive waste from the AREVA reprocessing plant in La Hague. The fact that those radionuclide releases result in a very small increase in radioactivity in oysters, especially compared with natural radioactivity, may be an explanation. However in this in situ approach, because of the complexity of the natural environment, we cannot rule out the possibility that other parameters may have masked the impact of radioactive discharges. Our data showed that, in the natural environment, mRNA levels of stress-inducible genes in oysters fluctuate seasonally. This provided knowledge of variations in these genes' expression levels, especially seasonal fluctuations and natural inter-individual variability. This was necessary preliminary work before we could tackle the "chronically low dose exposure" issue. On the basis of this knowledge, the next step is now to artificially expose oysters to radionuclides under laboratory conditions and to study the effects of chronic exposure to radionuclides on the mRNA levels of stress inducible genes.

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FIGURE CAPTIONS

Table 1: Primer sequences and accession number.

Table 2: Whole soft tissue concentrations of artificial gamma emitter radionuclides in oysters from St-Vaast (STV), expressed in $Bq.kg^{-1}$ dry weight ± measurement uncertainty. Values below the detection limit are indicated by the symbol -.

Table 3: Whole soft tissue concentrations of transuranic radionuclides in oysters from Carnac (CRC), Cancale (CCL) and St-Vaast (STV), expressed in $Bq.kg^{-1}$ dry weight ± measurement uncertainty. Values below the detection limit are indicated by the symbol <.

Table 4: Correlation matrix on axes 1 and 2. In bold: values>0.7. The correlation is given by the angle between the descriptor and axis, the closer to unity (unsigned value), the stronger the correlation. The five genes explained component one well.

Table 5: Matrix of the test values for the illustrative parameter. For each axis and each illustrative parameter, the test value expresses the statistical significance of the parameter's coordinate on this axis. In bold: significant values at the level of significance alpha = 0.05, which implies absolute values>2.0.

Figure 1: Map showing the AREVA reprocessing plant and the sampling locations along the French coast.

Figure 2: Monthly values of seawater temperature in Goury-Harbour near St-Vaast. In grey: the period with a temperature <10°C. The grey bands indicate the periods with seawater temperature below 10°C.

Figure 3: Hsp70 (a), Hsc72 (b), Hsp90 (c), SOD (d) and MT (e) mRNA levels in gills (dotted line) and whole soft tissues (solid line) in oysters collected monthly in STV between January 2004 and September 2005. Symbol * means a statistically significant difference ($p<0.05$) between the two paired data (Mann-Whitney test, n=4 pools of 5 individuals) and the associated value corresponds to their ratio. The grey bands indicate the periods displaying seawater temperature below 10°C.

Figure 4: mRNA levels of Hsp70 (a), Hsc72 (b), Hsp90 (c), SOD (d) and MT (e) in oysters collected quarterly in CRC (white), CCL (hatched), ASN (grey) and STV (black). * placed above a bar for locations exposed to radionuclide releases (STV, ASN, CCL) means a statistically significant difference in mRNA level ($p<0.05$) between this location and the reference site (CRC) (Mann-Whitney test, n=4 pools of 5 individuals).

Figure 5: Correlation circle for the variables (genes) on axes 1 and 2.

Figure 6: Principal component analysis biplot. The size of the symbol is proportional to the contribution (squared cosines of the variable). Axis 1 clearly separates data as a function of time and this first axis showed a clear strong contrast between winter and summer data.

TABLES

Table 1: Primer sequences and accession number.

Gene (or rRNA)	GenBank accession #	Forward Primer	Reverse Primer
*actin	AF026063	5′ GCCCTGGACTTCGAACAA 3′	5′ CGTTGCCAATGGTGATGA 3′
*18S	AB064942	5′ CGGGGAGGTAGTGAC GAA 3′	5′ ACCAGACTTGCCCTCCAA 3′
*GAPDH	AJ544886	5′ TTGTCTTGCCCCTCTTGC 3′	5′ CGCCAATCCTTGTTGCTT 3′
Hsp70	AJ305315	5′ AGCAAGCCAGCACAGCA 3′	5′ GCGATGATTTCCACCTTC 3′
Hsc72	AF144646	5′ GAGGATCGCAGCCAAGAA 3′	5′ TATCGCCCTCGCTGATCT 3′
Hsp90	AJ431681	5′ GGAGAGCAAAACCCTCACC 3′	5′ TGGCAATGGTTCCAAGGT 3′
**SOD	AJ496219	5′ AACCCCTTCAACAAAGAGCA 3′	5′ TTTGGCGACACCGTCTTC 3′
***MT	AJ243263 AJ242657	5′ GGACCGGAAAACTGCAAA 3′	5′ CCAGTGCATCCTTTACCACA 3′

* used to normalize mRNA.

** The amino acid translation of the Genbank #AJ496219 sequence is highly homologous to a Cu-Zn SOD.

*** This pair of primers did not discriminate between two identified members of oyster MTs: MT1 (accession # AJ243263) and MT2 (accession # AJ242657).

Table 2: Whole soft tissue concentrations (in $Bq.kg^{-1}$ dry weight) of artificial gamma emitter radionuclides in oysters from St-Vaast.

Month	^{60}Co	^{137}Cs
Jul-02	0.18 ± 0.17	-
Aug-02	0.19 ± 0.14	-
Sep-02	0.18 ± 0.17	-
Dec-02	-	0.50 ± 0.40
Apr-03	0.20 ± 0.14	0.16 ± 0.14
Jul-03	-	0.34 ± 0.13
Aug-03	0.14 ± 0.12	-
Sep-03	0.12 ± 0.11	-
Nov-03	0.14 ± 0.12	-
Dec-03	0.16 ± 0.13	-
Mar-04	0.23 ± 0.14	-

Table 3: Whole soft tissue concentrations (in $Bq.kg^{-1}$ dry weight) of transuranic radionuclides in oysters from Carnac (CRC), Cancale (CCL) and St-Vaast (STV).

Location	Month	^{241}Am	^{244}Cm	^{238}Pu	$^{239}Pu + ^{240}Pu$
CRC	Jun-04	0.0011 ± 0.0003	< 0.0001	0.0004 ± 0.0002	0.0101 ± 0.0009
CCL	Jun-04	0.0167 ± 0.0019	0.0018 ± 0.0004	0.0086 ± 0.0011	0.0280 ± 0.0024
STV	Aug-02	0.0289 ± 0.0051	0.0042 ± 0.0019	0.0073 ± 0.0020	0.0153 ± 0.0029
STV	Dec-02	0.0042 ± 0.0005	0.0008 ± 0.0002	0.0100 ± 0.0020	0.0255 ± 0.0033
STV	Jun-04	0.0152 ± 0.002	0.0021 ± 0.0006	0.0105 ± 0.0140	0.0273 ± 0.0024

Table 4: Correlation coefficient between mRNA levels in STV and temperature of the seawater in Goury Harbour.

r	Whole soft tissue	Gills
Hsp70	-0.50	**-0.74**
Hsc72	-0.24	-0.53
Hsp90	-0.53	**-0.77**
SOD	-0.18	**-0.68**
MT	**-0.64**	**-0.66**

Table 5: Correlation matrix on axes 1 and 2.

Variables	Correlation Variable-Factor (axis 1)	Correlation Variable-Factor (axis 2)
Hsp70	**-0.87**	0.33
Hsc72	**-0.83**	-0.03
Hsp90	**-0.71**	-0.52
SOD	**-0.81**	0.47
MT	**-0.78**	-0.37

Table 6: Matrix of the test values for the illustrative parameter.

Descriptor	Test-value (axis 1)	Test-Value (axis 2)
STV	0.0	0.1
CCL	-1.6	-0.8
ASN	0.4	0.8
CRC	1.3	-0.1
March 2004	-0.3	-0.2
June 2004	**2.3**	-0.4
Sept 2004	**4.1**	**-2.9**
Jan 2005	**-4.1**	0.4
March 2005	**-6.1**	0.9
June 2005	**3.1**	**2.4**

Figure 1: Map showing the AREVA reprocessing plant and the sampling locations along the French coast.

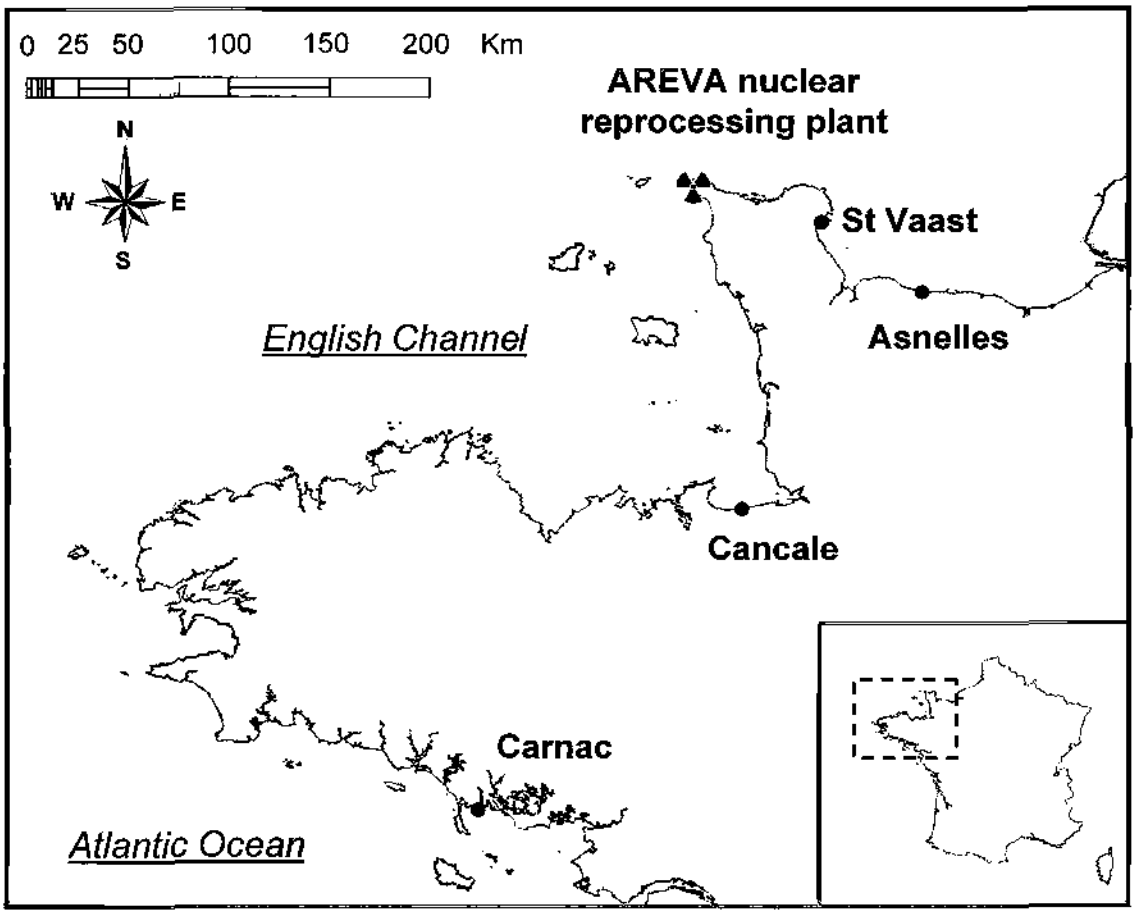
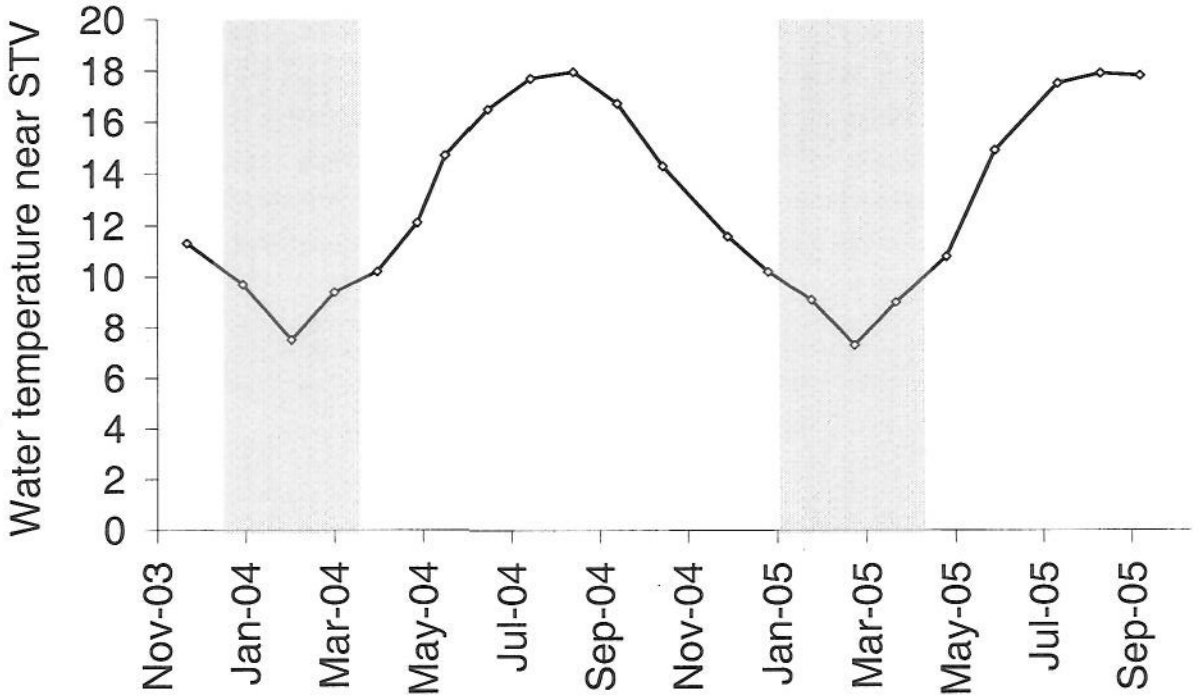


Figure 2: Monthly values of seawater temperature in Goury-Harbour near St-Vaast.



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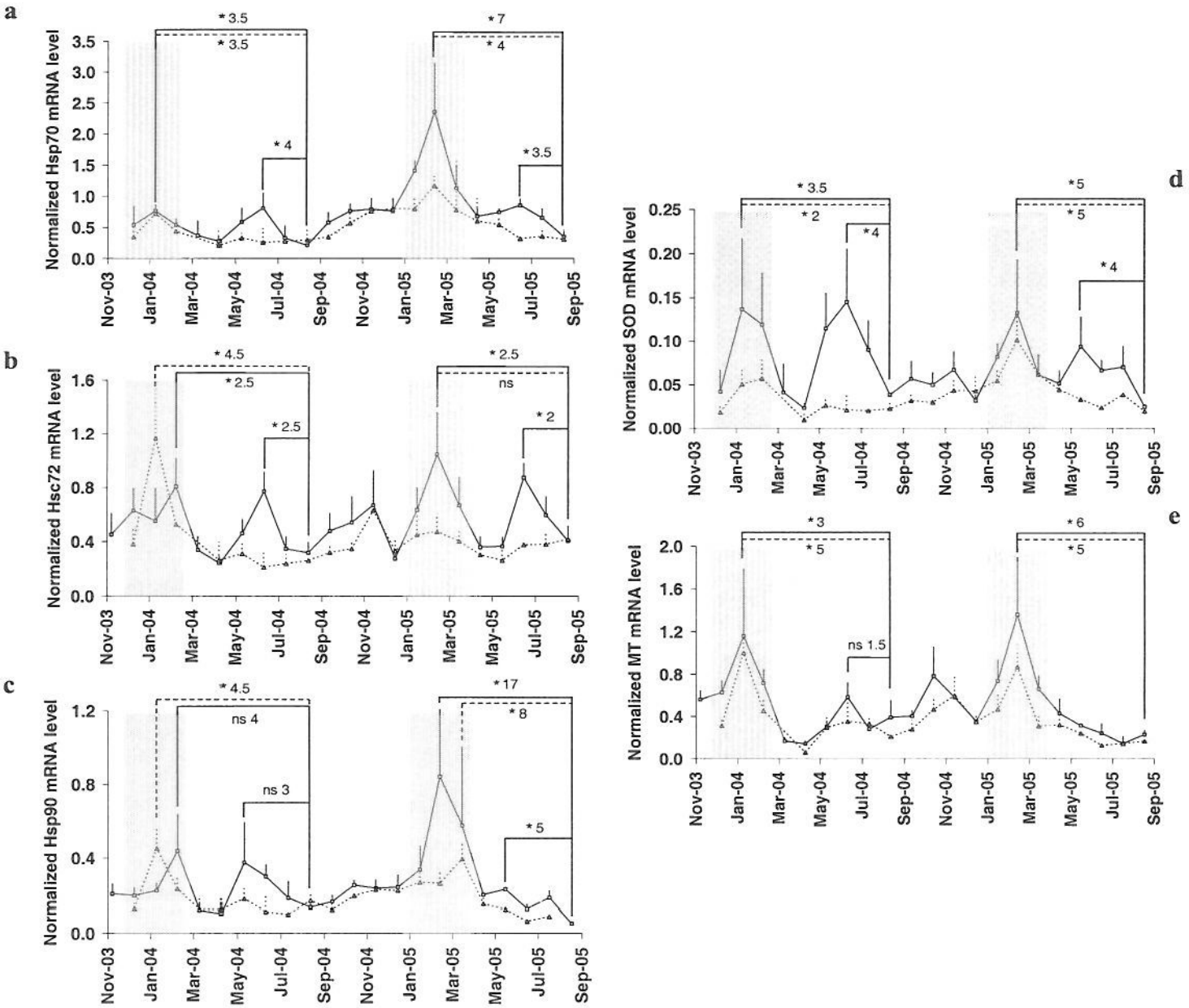

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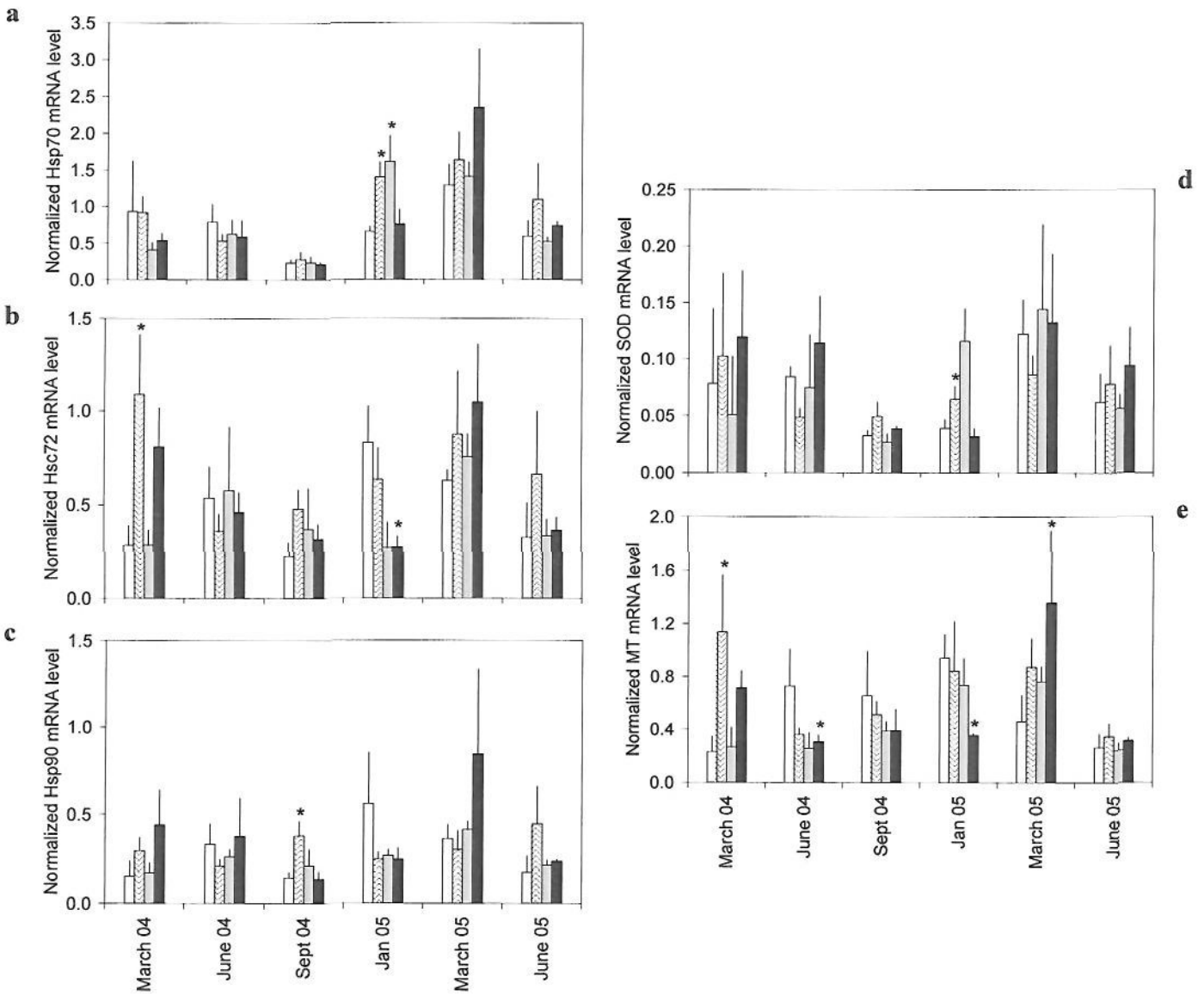

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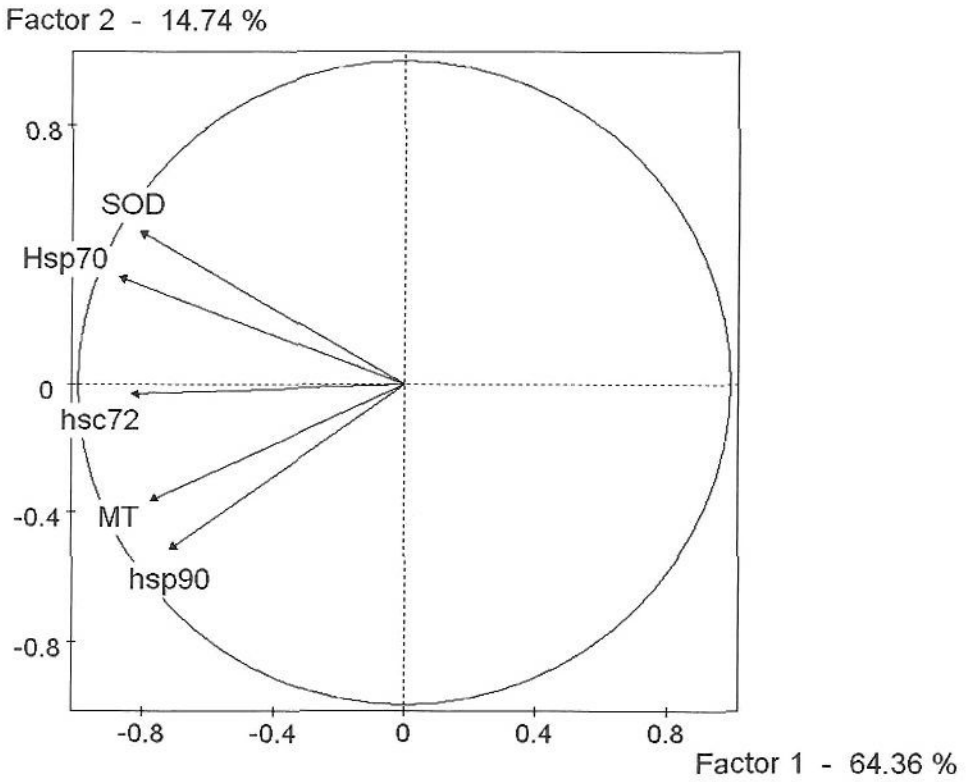

Figure 6: Principal component analysis biplot.