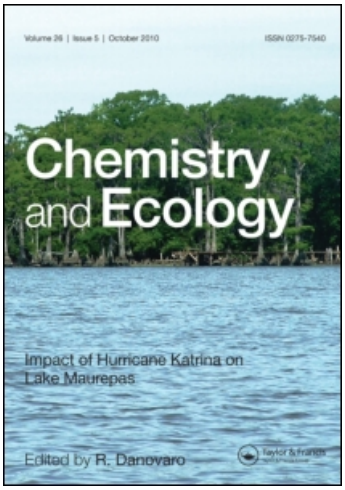


Chemistry and Ecology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713455114

HSP70 gene expression in sipunculids: A new biomarker for monitoring marine deposits

R. Bedini[a]; S. Pucciarelli[b]; R. Gatta[b]; A. Nannelli[a]; C. Miceli[b]
[a] Institute of Marine Biology and Ecology, Piombino, Italy [b] Department of Molecular, Cellular and Animal Biology, University of Camerino,

To cite this Article Bedini, R. , Pucciarelli, S. , Gatta, R. , Nannelli, A. and Miceli, C.(2004) 'HSP70 gene expression in sipunculids: A new biomarker for monitoring marine deposits', Chemistry and Ecology, 20: 3, 345 — 352
To link to this Article: DOI: 10.1080/02757540310001629143
URL: http://dx.doi.org/10.1080/02757540310001629143

Chemistry and Ecology
June 2004, Vol. 20 (Supplement 1), pp. S345–S352

Taylor & Francis
Taylor & Francis Group

HSP70 GENE EXPRESSION IN SIPUNCULIDS: A NEW BIOMARKER FOR MONITORING MARINE DEPOSITS

R. BEDINI[a,*], S. PUCCIARELLI[b], R. GATTA[b], A. NANNELLI[a] and C. MICELI[b]

[a]*Institute of Marine Biology and Ecology, Piazza Bovio 3/4, 57025 Piombino, Italy;*
[b]*Department of Molecular, Cellular and Animal Biology, University of Camerino*

Sipunculids have been used as ecological bioindicators in the areas of the harbour of Piombino (Livorno, Italy), the near pier in Follonica (Grosseto) and the harbour of Portoferraio (Elba Island, Livorno). The species of the collected samples were identified by molecular analysis of the 18S rRNA sequence: a genetically homogeneous population of *Phascolosoma granulatum* Leuckart, 1828, was found to be present in all the three analysed areas. The sipunculids, collected from different sites of the three areas exposed to identical environmental conditions (depth, temperature and solar radiation), were analysed in parallel to quantify the expression of HSP70, a molecular biomarker of ecological perturbation and contamination. This analysis showed that the largest amount of HSP70 is expressed in specimens taken from sites of the Piombino harbour, an area particularly exposed to industrial waste. The expression of an isoform larger in size than the conventional HSP70 was highlighted in these specimens.

Keywords: Biomarker; Biomonitoring; Sipunculids; Heat-shock proteins; Metallotioneines

1 INTRODUCTION

In the context of a study on environmental monitoring in harbour areas, methodologies for the characterization of sipunculids (see Fig. 1) as new ecological bioindicators, to be used in synergy with, or as an alternative to, other classic 'sentry' organisms were carried out. The type of feeding of sipunculids makes them particularly suitable as bioindicators (Pancucci-Papadopoulou *et al.*, 1999). They are, in fact, principally 'deposit feeders' instead of 'filter feeders' such as mussels. Thus, they filter sandy sediments that include deposits of pollutants and contaminated substances. They therefore, adopting as feeding strategy an unselective filtering system, absorb a fair amount of inorganic compounds, including heavy metals (Pb, Hg, Cr, etc.) that are dangerous to metabolism (Magnum and Burnett, 1987), and they can also ingest poorly soluble organic pollutants, such as organotin compounds.

Many studies emphasize a relationship between the presence of heavy metals and other contaminants and the production of heat-shock proteins (HSPs) by the organism, in order to cope with the damaging effects caused by these elements. The proteins induced by environmental changes, especially HSPs, are very interesting because of the numerous prospective applications, above all in medical and environmental matters, and particularly in the

* Corresponding author. Tel.: +39 0565225196; Fax: +39 0565227021; E-mail: bedini@biomare.it

ISSN 0275-7540 print; ISSN 1029-0370 online
DOI: 10.1080/02757540310001629143

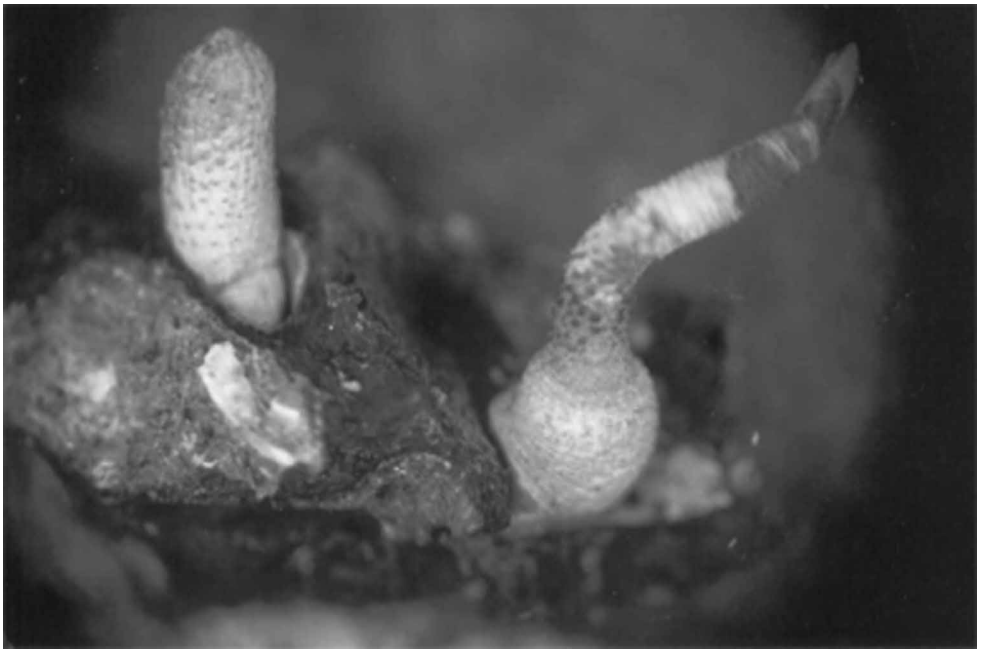


FIGURE 1 *Phascolosoma granulatum* Leuckart,1828 (magnification 16×).

biomonitoring of environments. One family of these environmental stress proteins, the HSP70, induced by a wide spectrum of stressful agents and conditions, provides a biological marker for environmental perturbations. Indeed, variations of the expression of these proteins can represent a diagnostic instrument of environmental contamination.

The study here presented at first involved sampling in places affected by a strong anthropic impact. Then the expression of HSP70 in specimens taken from different sites was monitored, as an attempt to establish whether sipunculids could be used as ecological bioindicators. A molecular approach was also carried out to determine the species of the analysed samples. For this purpose, sequence analyses of the gene codifying the 18S ribosomal subunit were carried out, as this gene is one of those best conserved during evolution and is usually considered to be of taxonomic value.

2 MATERIAL AND METHODS

2.1 Monitoring Areas

Samples of sipunculids were collected in the harbours of Piombino (Livorno) and Portoferraio (Elba Island), and at the 'Solmine' pier in Follonica (Grosseto). These three areas are zones of considerable environmental interest showing different features. The harbour of Portoferraio is destined only for the transit of ferryboats that link it to Piombino. The harbour of Piombino is destined for the loading and unloading of goods (coal and iron ore) and is subjected to sewage discharge from the Magona steelworks. To have a larger picture of the situation within this harbour area, three different sampling sites were chosen: a 'Deep water' site (whose samples were called Piombino IA), that is more exposed to ferryboat transit and is relatively far from the siderurgical zone; the 'Lucchini' area (Piombino IB), where there is industrial waste from

the steelworks; the 'Magona' pier (Piombino II), occupied by coal and iron ore depots. The 'Solmine' pier in Follonica was chosen because of the presence of an industrial waste different from that of Piombino and to verify if the population of sipunculids exposed to a differing kind of environmental stress was genetically close to that taken from the other areas.

2.2 Taking of Samples

Diving with scuba equipment, the specimens were taken from submerged walls in the various sites concerned by scratching surfaces of (30×30) cm^2 (Cinelli *et al.*, 1977; Sarà *et al.*, 1978), at a depth of -1, -3 and -5 meters in order to obtain samples at three levels, near the surface, at medium depth, and near the bottom. Once collected, the samples were immediately frozen in liquid nitrogen until the analysis was performed. The specimens, still frozen, were divided into two portions: the front part was used to evaluate the degree of genetic homogeneity of the population studied and the back to verify the presence of stress proteins. The homogenisation of both portions was made in a cold room with a Pyrex surge tank mortar (Wheaton, USA).

2.3 DNA Purification

For DNA extraction the homogenisation was carried out in a NDS solution (10 mM Tris/HCl, 0.1% SDS, 0.5 M EDTA, pH 9.5). The homogenised material was subsequently incubated for 2 hrs at 55 °C in NDS containing 200 μg/ml of proteinase K. It was extracted first with a volume of phenol/chloroform (1:1) and then with a volume of chloroform. The DNA was precipitated with 0.4 M LiCl and two volumes of absolute ethanol at −20 °C for 1 hr and later suspended in water and precipitated again with 13% PEG and 15 mM $MgCl_2$, for 30 min at room temperature (Pucciarelli and Miceli, 2002).

2.4 Amplification Techniques by Polymerase Chain Reaction and Sequencing Techniques

Polymerase chain reactions (PCR) were carried out with a DNA Thermal Cycler (Perkin-Elmer Cetus Instrument). The strategies consisted of a first step of denaturation at 94 °C, a second step of annealing at 45 °C, and a final one of elongation at 72 °C. Each step lasted 1 min and the whole cycle (Steps 1, 2 and 3) was repeated 30 times (Pucciarelli and Miceli, 2002). PCR products were sequenced using a Perkin-Elmer kit performing 25 cycles of 1 min of denaturation at 96 °C, 15 secs of annealing at 50 °C, and 4 min of primer extension at 60 °C. After precipitation in ethanol to remove unassembled labelled nucleotides, the results of these reactions were analysed by the ABI 373 automatic DNA sequencer (Perkin-Elmer).

2.5 Sequences Analysis and Reconstruction of Phylogenetic Trees

Sequences were analysed and aligned with the CLUSTAL X programme (Aiyar, 2000). Dendrograms were built with the TREECON 1.15 programme, using the Neighbor-Joining method (Saitou and Nei, 1987). The significance of trees was evaluated by employing 1000 bootstraps.

2.6 Cellular Extract Preparation and Western Blot Analysis

For cell extract preparation, homogenisation was carried out in TEM buffer (20 mM Tris, 1 mM EGTA, 1 mM $MgCl_2$, pH 7.5) including protease inhibitors (2 mM PMSF, 2 mM *o*-fenantroline, 10 mg/ml pepstatine, 1 mM DTT) (Pucciarelli *et al.*, 1997). The fraction for the HSP70 study was diluted in an equal volume of SDS loading buffer (Laemmli, 1970), boiled for 5 min and then preserved at $-20\,^{\circ}C$. Electrophoresis on polyacrylamide gel in SDS (SDS–PAGE) and Western blot were performed following classical techniques (Laemmli, 1970; Green *et al.*, 1995). Nitrocellulose membrane was incubated with anti-HSP70 antibodies (Sigma) and, apart, with antibodies (Sigma) against α-tubulin (a protein that has been found not to be affected by stress conditions in its expression (La Terza *et al.*, 2001), as a term of comparison to normalise the amount of loaded proteins.

The intensity of bands obtained from Western blot analyses was quantified with the Kodak Digital Science program; these values were normalised with those obtained for α-tubulin.

3 RESULTS AND DISCUSSION

3.1 Determination of the Species

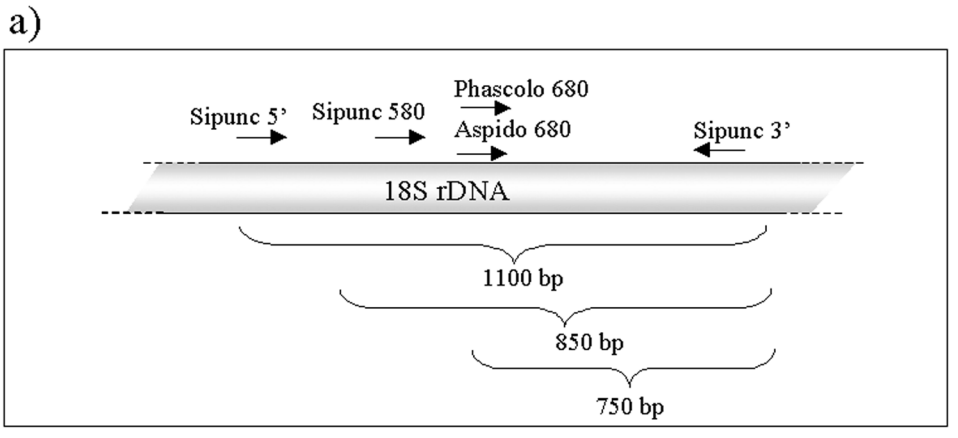
In order to identify the species of the samples, the 18S rRNA codifying sequence was analysed. Using the CLUSTAL X program, the known sipunculid 18S rRNA sequences, already present in the data bank, were aligned. Oligonucleotides corresponding to regions conserved in all sipunculids and specific for a single genus were designed (see Tab. I). These were used as primers in the strategies of gene amplification through PCR (as shown in Fig. 2(a)).

The results of the PCR and in particular of the strategies numbered 3 and 4 indicate that all the species analysed (for which only one representative analysis is shown in Fig. 2(b)) belong to the genus *Phascolosoma.* This datum was also confirmed by the sequences of PCR products obtained in the reaction number 1 (the longer fragment). Furthermore, the alignment of the obtained sequences with those reported in the data bank indicate that all the samples analysed belong to the species *Phascolosoma granulatum* Leuckart, 1828, as already identified by an observation of anatomic features, with very small and probably individual variations (data not shown). The sequences obtained were also used to construct a phylogenetic tree, built by adding the other sipunculid sequences available in the data bank and other invertebrate sequences (see Fig. 3). From this analysis the monophyletic origin of all sipunculids that diverged in parallel to mollusca, annelida and priapulida can be highlighted.

TABLE I List of oligonucleotides used as primers for PCR strategies.

Oligonucleotide name	*Sequence 5′–3′*	*Specificity*
SIPUNC 5′	GGAAGAGCGAGTTTATTAGATC	All sipunculids
SIPUNC 3′	TCTCGTGTGCATTCCATGCAC	All sipunculids
SIPUNC 590	GCCGCGGTAATTCCAGCTCCA	All sipunculids
ASPIDO 680	GCCTCGCGGCGGTTACTGCCC	*Aspidosiphon*
PHASCOLO 680	TAGCTCCCTTGCCGGCAACTG	*Phascolosoma*

Note: Oligonucleotides SIPUNC 5′, SIPUNC 3′ and SIPUNC 590 were designed on the basis of preserved regions of codifying sequences of the 18S rRNA of sipunculids taken from the data bank. The oligonucleotides ASPIDO 680 and PHASCOLO 680 were designed on the basis of genus-specific regions of *Phascolosoma* and *Aspidosiphon*.



b)

PCR strategy	5' primer	3' primer
1	Sipunc 5'	Sipunc 3'
2	Sipunc 580	Sipunc 3'
3	Phascolo 680	Sipunc 3'
4	Aspido 680	Sipunc 3'

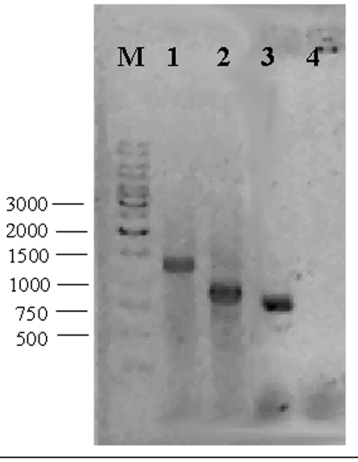


FIGURE 2 (a) Schematic representation of the relative location of oligonucleotides used as primers in PCR strategies on the 18S rRNA codifying gene and the expected sizes of PCR products, indicated in base pairs (bp). (b) On the left, combination of the primers used in the different PCR strategies that were numbered from 1 to 4; on the right, ethidium bromide staining of an agarose gel showing the amplified DNA products obtained from the PCR strategies carried out using as template DNA extracted from sipunculids sampled in the different areas. The molecular size marker lane is indicated by 'M' and markers are reported in bp on the left of the gel.

3.2 Analysis of Stress-Induced Proteins

Cell extracts of sipunculids were analysed by Western blot for the presence of the HSP70, using commercial anti-HSP70 antibodies (SIGMA). The blot membrane was incubated with the anti-HSP70 antibodies. Then, after removal of the previous antibodies, it was incubated with the α-tubulin monoclonal antibodies to normalize the quantity of loaded proteins (see Fig. 4).

Through this analysis it was shown that in all the samples bands of equal molecular weight of about 70 KDa could be found, as expected on the basis of the fact that the HSP70 gene is usually essential in all organisms and therefore always present (the HSP70 bands for samples XXII and XVI are scarcely visible in Fig. 4, but present in the original analyses). Particularly interesting is the result of samples XVI, XVII, XX and XXII, in which additional bands of higher molecular weight are present. These larger isoforms

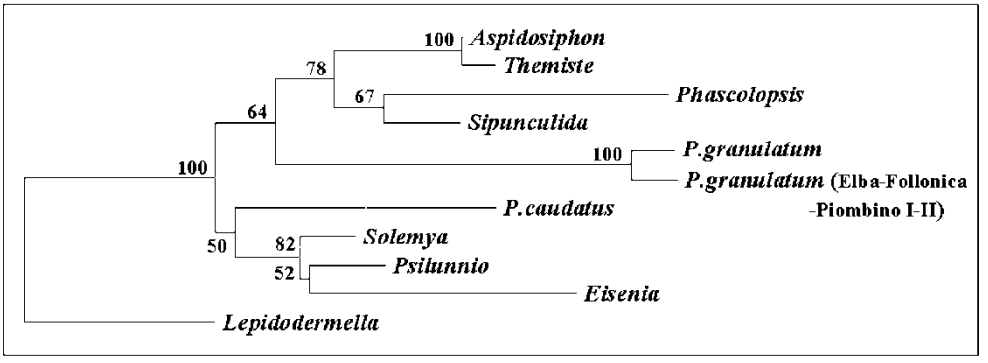


FIGURE 3 Phylogenetic Dendrogram constructed by alignments of the 18S rRNA codifying sequences of sipunculids taken in Piombino, Follonica and Elba Island with those of other sipunculids obtained from the data bank (*Phascolosoma granulatum*, X79874; *Aspidosiphon misakiensis*, AF119090; *Themiste alutacea*, AF119075; *Phascolopsis gouldi*, AF342796), and of organisms belonging to different phyla (*Priapulus caudatus* X80234, priapulid; *Eisenia fetida* X79872, earthworm; *Solemya velum* AF120524, bivalve; *Psilunnio littoralis* AF120536, bivalve). Sequences were analysed with the CLUSTAL X program. As an outgroup the gastrotrich *Lepidodermella squammata* (U29198) was used.

(called HSP79) resemble the HSP70 isoforms induced by specific stresses in other organisms. In fact, both in protozoa and in invertebrates (Lyons and Johnson, 1998; Jayasena *et al.*, 1999) inducible forms of HSP70 were found that differ mainly in the number of repetitions of a fairly well conserved domain of about seven aminoacids. In inducible forms, these domains were repeated up to 10-fold more than in non-inducible forms. The results reported here suggested that the larger isoforms correspond to the inducible protein in sipunculids as well, activated in its transcription, and therefore in its translation, only in the presence of environmental contaminants. A histogram presentation has been built up with the expression values of the two HSP70 isoforms calculated according to the intensity of the bands and normalised to the values obtained with the anti-α-tubulin antibodies. From this analysis it appears that the isoform HSP79 is mainly expressed in those samples taken from the areas of Piombino IB and II, particularly exposed to industrial waste and in which the concentration of heavy metals and organic pollutants in the sediments has been reported to be greater than in the harbour of Portoferraio (for example, in the areas of Piombino IB and II chrome concentration was 68.75 ppm as opposed to the concentration of 14.47 ppm found in the

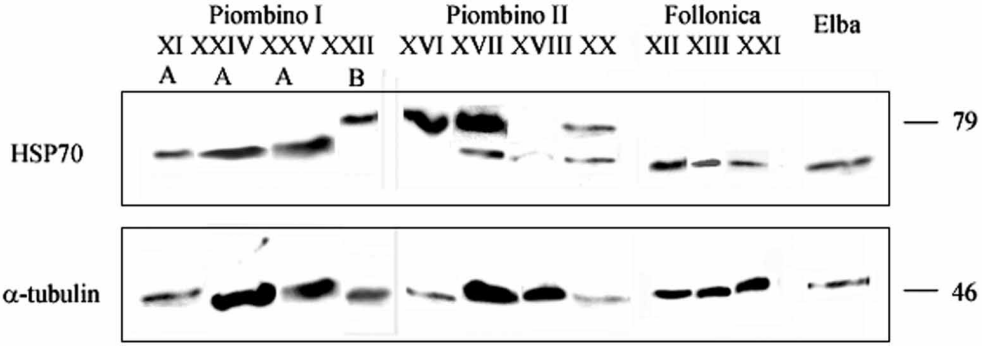


FIGURE 4 Western blot of sipunculid extracts, incubated with the monoclonal antibodies anti-HSP70 and with the monoclonal anti-α-tubulin antibodies. The standard molecular weights are indicated at one side.

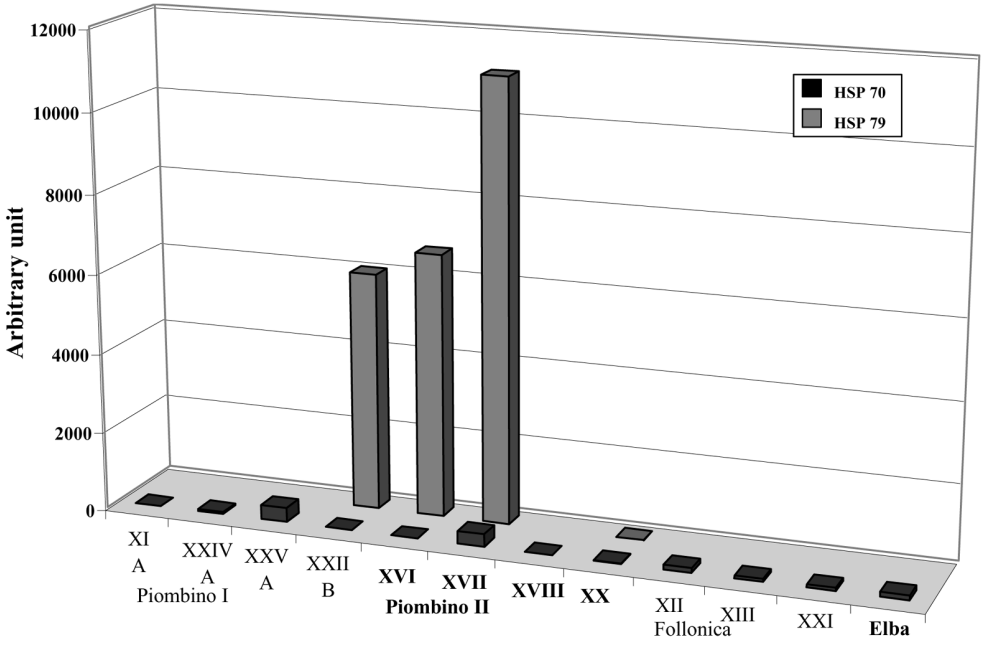


FIGURE 5 Histogram showing the expression values of the two HSP70 isoforms, the conventional one (HSP70) in black, corresponding to the lower band taken from the Western blot in Fig. 4, and the larger one (in grey), called HSP79, corresponding to the higher band in Fig. 4. The expression values were calculated according to the intensity of the bands and successively normalised by comparison to the bands obtained with the anti-α-tubulin antibodies.

sediments collected in Portoferraio. See as data references Greenpeace, 1999; ARPAT, 2000; ICRAM, 2000). These same Piombino samples are also shown to express the largest amount of HSP70, considering both isoforms. None of the samples taken from the areas of Follonica and Elba Island showed the expression of this particular larger HSP70 isoform.

In conclusion, the data reported here show that sipunculids can be used as bioindicators for environmental monitoring in harbour areas. A procedure has been defined that permits the identification of the species of each individual sample through PCR analysis. Furthermore, it permits in the same sample to analyse the expression of the HSP70 environmental biomarker.

References

Aiyar, A.(2000). The use of CLUSTAL W and CLUSTAL X for multiple sequence alignment. *Methods in Molecular Biology*, **132**, 221–241.

ARPAT, Servizio Sub-Provinciale di Piombino (2000). Analisi dei sedimenti prelevati nel Porto di Portoferraio. Trasmissioni dei risultati. Prot. 2096.

Cinelli, F., Fresi, E., Mazzella, L., Pansini, M., Pronzato, R. and Svoboda, A. (1977). Distribution of phyto- and zoocenosis along a light gradient in a superficial marine cave, in Keagan, B. F., O'Ceidigh, P. and Boaden, P. S. S. (eds.), *Biology of benthic organisms.* Pergamon Press, Oxford, pp. 173–183.

Green, J. M., Gu, L., Iftilkovits, C., Kaumaya, P. T., Conrad, S. and Pierce, S. K. (1995). Generation and characterization of monoclonal antibodies specific for members of the mammalian 70-kDa heat shock protein family. *Hybridoma*, **14**, 347–354.

Greenpeace Italia (1999). Valutazione del progetto di scarico a mare dei fondali dell'area di evoluzione del porto di Piombino. A cura di Fabrizio Fabbri.

ICRAM (2000). Dragaggio Porto di Piombino. Prot. 68428.

La Terza, A., Papa, G., Miceli, C. and Luporini, P. (2001). Divergence between two Antartic species of the ciliate *Euplotes, E. focardii* and *E. nobilii*, in the expression of heat-shock protein 70 genes. *Molecular Ecology*, **10**, 1061–1067.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Lyons, R. E. and Johnson, A. M. (1998). Gene sequence and transcription differences in 70 KDa heat shock protein correlate with murine virulence of *Toxoplasma gondii*. *International Journal of Parasitology*, **28**, 1041–1051.

Jayasena, S. M., Chandrasekharan, N. V. and Karunanayake, E. H. (1999). Molecular characterization of a HSP70 gene from the filarial parasite *Setaria digitata. International Journal of Parasitology*, **4**, 581–591.

Magnum, C. P. and Burnett, L. E. (1987). Response of sipunculid hemerythrins to inorganic ions and CO_2. *Journal of Experimental Zoology*, **244**, 59–65.

Pancucci-Papadopoulou, M. A., Murina, G. & Zenetos, A. (1999). The phylum sipuncula in the Mediterranean Sea. *Monographs on Marine Sciences*, **2**, 11–93.

Pucciarelli, S., Ballarini, P. and Miceli C. (1997). Cold-adapted microtubules: characterization of tubulin posttranslational modifications in the Antarctic ciliate *Euplotes focardii. Cell Motility and Cytoskeleton*, **38**, 329–341.

Pucciarelli, S. and Miceli, C. (2002). Characterization of the cold-adapted α-tubulin from the psychrophilic ciliate *Euplotes focardii. Extremophiles*, **5**, 385–389.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: a new method for reconstruction of phylogenetic trees. *Molecular Biology and Evolution*, **4**, 406–425.

Sarà, M., Balduzzi, A., Boero, F., Pansini, M. and Pronzato, R. (1978) Analisi di un popolamento bentonico di falesia del Promontorio di Portofino: dati preliminari. *Bollettino dei Musei e degli Istituti Biologici dell'Università di Genova*, **46**, 119–137.