

## Physiological and Cellular Responses in Two Populations of the Mussel *Perna perna* Collected at Different Sites from the Coast of São Paulo, Brazil

Denis Moledo de Souza Abessa<sup>1,2\*</sup>, Leticia Pires Zaroni<sup>1</sup>, Eduinetty Ceci Pereira Moreira de Sousa<sup>1</sup>, Marcia Regina Gasparro<sup>1</sup>, Camilo Dias Seabra Pereira<sup>1,3</sup>, Bauer Rodarte de Figueredo Rachid<sup>1</sup>, Michael Depledge<sup>4</sup> and Rebecca Susan King<sup>4</sup>

<sup>1</sup>Instituto Oceanográfico; Universidade de São Paulo; Praça do Oceanográfico, 191; 05508-900; São Paulo - SP - Brazil; <sup>2</sup> Universidade Estadual Paulista; Campus do Litoral Paulista; Praça Infante Dom Henrique, s/n, 11330-900; São Vicente - SP - Brazil; <sup>3</sup> Universidade Santa Cecília; Rua Oswaldo Cruz, 266; 11045-100; Santos - SP - Brazil; <sup>4</sup>Plymouth Environmental Research Centre; University of Plymouth; Drake Circus; Plymouth; PL4 8AA -UK

### ABSTRACT

*The physiological conditions of mussels from Ubatuba and Santos and also of organisms transplanted from Ubatuba to Santos were studied by using different techniques. Assays for lysosomal stability were conducted on the haemolymph. Heart rate activity was monitored for 6h. The embryonic development of larvae obtained from the collected mussels was analysed. For all the compared groups of mussels, no significant differences were observed for the cardiac activity monitoring and the embryonic bioassays. The mean Neutral Red (NR) retention time was similar for the animals from Santos and Ubatuba, whereas the organisms transplanted to Santos showed a reduction in the retention time of the dye, indicating damage in the lysosomal membranes. These differences were possibly due to environmental factors, but further investigations are required to confirm this hypothesis.*

**Key words:** *Perna perna*, neutral red retention assay, capmon system, embryonic assay, biomarkers, physiology

### INTRODUCTION

Since the 1970's, marine ecotoxicology has evolved from classical approaches, such as lethality assays (Reish, 1987), to the development of biochemical and physiological assays, adapted from human toxicology (Fossi et al., 2000). These new tools have been used for assessing early alterations due to contaminants, before irreversible effects or death occurs, therefore providing much more effective protection to the ecosystems (Payne et al., 1996). These early biological responses to

environmental chemicals are typically termed biomarkers.

Biomarkers have the advantage of being rapid, easy, highly sensitive and inexpensive. Techniques are available for evaluating stress at different levels of biological organization, from nuclear to whole individual level, including DNA stability, changes in enzymatic activity, modification of cells/tissues properties and other physiological alterations (Wells et al., 1998). Multi-level studies can produce more reliable information on the conditions of the studied organisms and provide the data necessary for the understanding of the

\* Author for correspondence

mode of action of pollutants, as well as the mechanistic comprehension of the processes related to intoxication and detoxication, which is especially desirable when assessing the early signs of stress.

In Brazil, the use of biomarkers in environmental evaluations is recent (Bainy et al., 2000). Few laboratories have the appropriate equipment and trained staff to perform such assays. More importantly, the protocols need to be adapted to species native to Brazil.

The brown mussel *Perna perna* (Bivalvia, Mytilidae) is native to the Brazilian Coast, also occurring in Venezuela, Uruguay, Argentina and South Africa (Rios, 1984). It is found on rocky shores of Southeast Brazil, forming dense natural colonies at the inter- and sub-tidal zones, and has economic importance, representing as a protein source as a significant source of income for economically disadvantaged coastal populations. Moreover, mussel farms are quickly spreading along the Brazilian coast, constituting an additional economic activity for many coastal cities. As a suspension feeder, *P. perna* may accumulate chemical compounds in its tissues. In Brazil, it has been used in studies regarding effects of xenobiotics at the biochemical (Bainy et al., 2000) and cellular levels (Zaroni et al., 2001), and also in bioaccumulation studies (Ferreira et al., 2000).

Recently, within the project "Early Warning, Chemical and Biological Markers of Pollution in Coastal Waters", a collaboration agreement was established between the University of Plymouth and the Oceanographic Institute of the University of São Paulo, in which some newly validated techniques for the cardiac activity assessment - CAPMON (Depledge and Andersen, 1990) and the Neutral Red retention assay (Moore, 1990) were introduced in Brazil, together with the necessary equipment and training.

In most macroinvertebrates, there is a close, positive relationship between aerobic metabolic rate and cardiac activity (Depledge and Lundebye, 1996). Thus, the measurement of heart rate and the regularity of beating provide a useful means of estimating physiological integrity. The CAPMON system (Depledge and Andersen, 1990) uses small infrared emitters/detectors applied to the external surface of the shells of the test animals. Infrared light is continuously emitted and the amount of light reflected back to the detectors varies according to the heart's cycle of action,

allowing thus the heart rate to be monitored. The detectors are interfaced with a PC, and by using appropriate software, the cardiac activity can be recorded and monitored for long periods of time.

Lysosomes are recognized target sites for most environmental contaminants, which can cause destabilization of the lysosomal membrane. The Neutral Red retention time assay evaluates the lysosomal membrane integrity, which can be used as an indicator of exposure to xenobiotics (Moore, 1990; Lowe et al., 1995; Cheung et al., 1997; Zaroni et al., 2001). It involves exposing cells to a coloured dye, which is taken up by the lysosomes. Healthy cells retain the dye for more time than damaged cells, in which the dye rapidly leaks out into the cytoplasm.

In addition to these previously mentioned methods, the success of embryonic development has been used in studies of the effects of xenobiotics on marine biota (Wedderburn et al., 2000; Zaroni et al., 2001) to the connective level (Dame, 1996). The larval viability may reflect the conditions of parental organisms exposed to *in situ* contaminants. Affected adult mussels show decrease in their offspring production, as observed by the reduction in the percent of well developed larvae in laboratory conditions after spawning induction.

In this study, these three methods, and their necessary adaptations, were used to compare the physiological conditions of two populations of *P. perna* from the State of São Paulo.

## MATERIALS AND METHODS

### Collection sites

The mussels were collected at rocky reefs situated at two sites along the State of São Paulo: Ponta Grossa and Palmas Island (Fig. 1). The collection sites differ essentially in two factors: proximity to contamination sources and influence of estuarine waters.

Ponta Grossa is situated on the North Shore of the state, in Ubatuba, a small city with a tourism-based economy. Except for its central area, which is urbanized, Ubatuba presents a very scattered human occupation, composed mostly of seasonally occupied houses. The Ponta Grossa collection site is considered relatively free of influence from human activities, and mussels collected from this

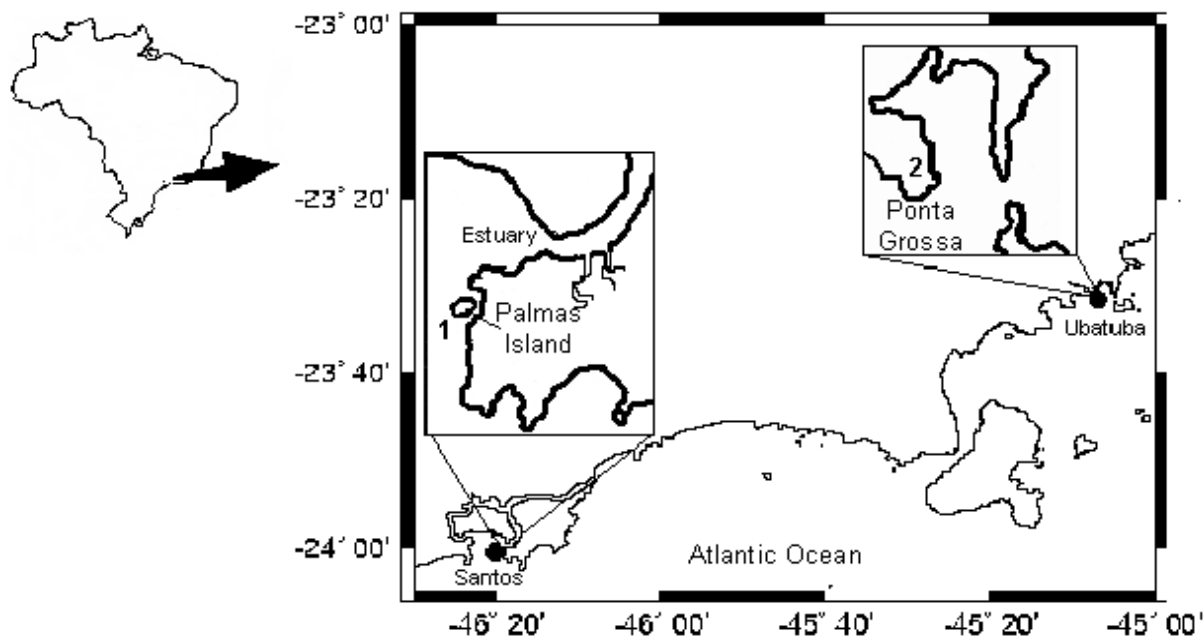
area were already considered healthy (Zaroni et al., 2001).

Palmas Island is located inside Santos Bay, on the central shore of São Paulo. The bay is part of the Santos Estuarine System (SES), an industrialized and urbanized region with some contaminated areas (Tommasi, 1979; Lamparelli et al., 2001; Abessa, 2002). Geographically, SES is divided in the estuaries of Santos and São Vicente and the Santos Bay. Sediments and organisms from both estuaries are contaminated by metals, PAHs and pesticides (Tommasi, 1979; Bonetti, 2000; Medeiros, 2000; Lamparelli et al., 2001; Abessa, 2002), as by industrial effluents as by domestic and other residues. The waters and sediments from the Santos Bay present low levels of contamination for the several chemicals (Lamparelli et al., 2001; Abessa, 2002). However, the influence of freshwater from the Estuary of Santos can also constitute a stressing factor for the mussels from Palmas Island.

#### Neutral Red retention assay

For this experiment, organisms were analysed immediately after collection and the assays were conducted at  $25 \pm 2$  °C. The physiological saline and NR stock solutions were prepared prior to the beginning of the assay, following the protocol

described by Moore (1990). Initially, 0.2-ml haemolymph from each mussel was collected from its posterior adductor muscle syringes containing 0.2-ml physiological saline solution and transferred to an Eppendorf tube. Immediately, 40- $\mu$ l haemocyte cells solution was pipetted and dropped onto a glass slide. The slides were placed into a dark and humid chamber and incubated for 15 min. During this interval, the NR working solution was prepared by the dilution of 10- $\mu$ l stock solution with 5-ml physiological saline solution. After incubation, 40- $\mu$ l NR working solution were dropped onto each slide. At the end of 15 min, the slides were quickly examined by microscopy. The cells were observed for structural abnormalities and for the retention time of the NR dye. The slides were observed at 15 min intervals, until the cells were considered affected. In each examination, the cell conditions were analysed and recorded in a table. A mean NR retention time was calculated for each group, and the means were compared by analysis of variance (ANOVA) followed by a Tukey's multiple comparison (Zar, 1984). Also, the percent of organisms showing effects at each reading time was calculated, aiming to compare the response behaviour.



**Figure 1** - Central and North coast of São Paulo, showing the mussel collection sites in Santos and Ubatuba.

### Cardiac activity monitoring system

Eight animals from each group were used. The sensors were attached in the appropriate position and then the mussels were transferred to 10-l aquaria in groups of 4 animals. They were acclimatised for 6h in the dark, at  $25 \pm 2$  °C, and then the cardiac activity was recorded. The experiments lasted 6h. During this period the animals were not disturbed. The heartbeat average for each animal was calculated, and for each group of mussels the mean heartbeat and its standard deviation were estimated. Then, the means were compared by ANOVA followed by Tukey's multiple comparison.

### Embryonic development assay

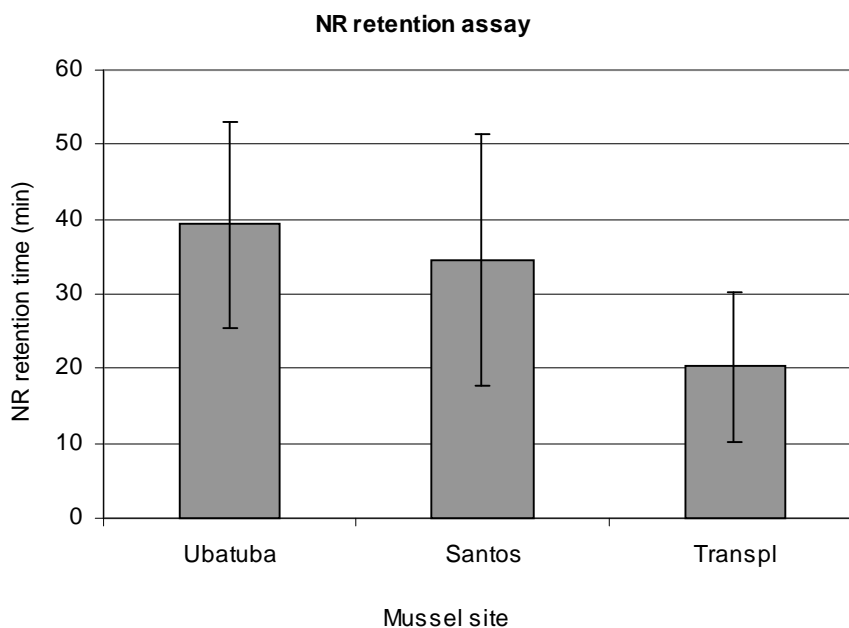
About 50 adult individuals were collected and their spawning was induced by thermal stimulation, according to the protocol recommended by ASTM (1992) for mussels, with minor adaptations for *P. perna* (Zaroni, 2000). The gametes from males and females were collected separately and transferred to glass beakers. The fertilisation was attained by adding 2ml of sperm solution to the ovules solution. Fertilised eggs were transferred to glass tubes containing clean, filtered seawater. Five replicates were used for each group. After 48 hours, the assay was finished and the first 100 larvae from each replicate were

counted. Larvae developed to D-phase were considered normal, whereas those presenting delay and/or morphological anomalies in their development were considered abnormal. The normal development means obtained for each group were compared by analysis of variance (ANOVA) followed by Tukey's multiple comparison.

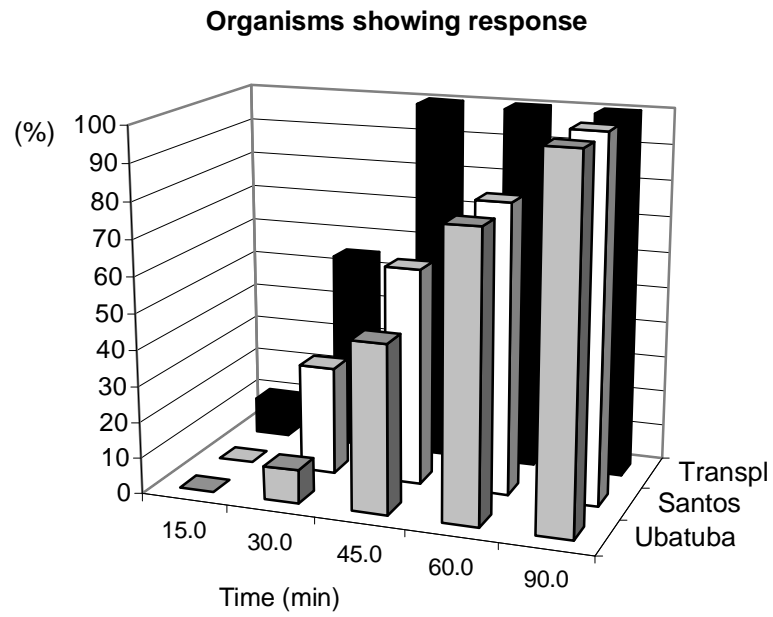
### Experimental Design

Initially, this study was designed to assess the conditions of mussels from the two study sites, and also to observe physiological modifications occurring as a consequence of transplanting organisms from Ponta Grossa to Palmas Island and *vice-versa*.

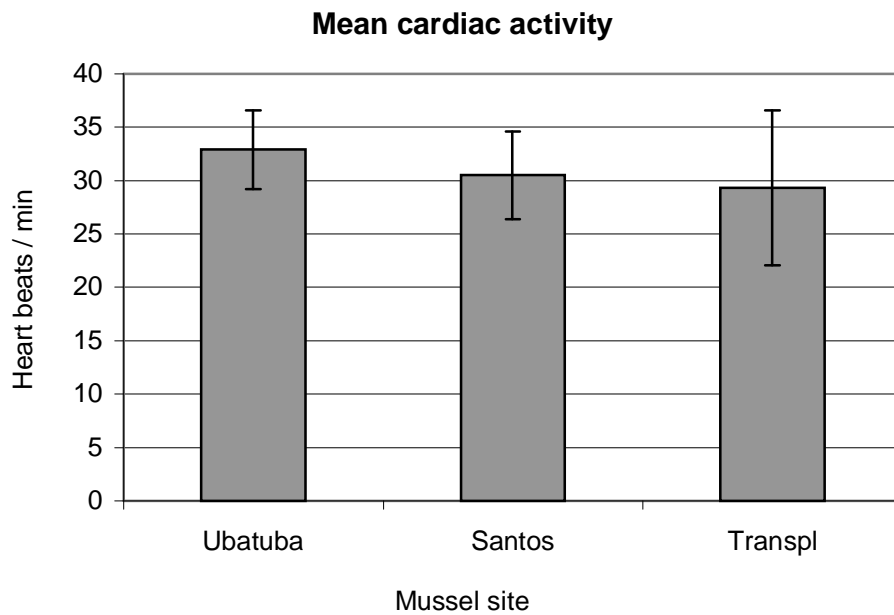
Two months prior to the beginning of the experiment, about 60 mussels were collected from natural populations in each site, placed into steel cages and immediately transplanted to the other site. In Palmas, the cages were attached to a pier, while in Ponta Grossa they were fasten to a cable with anchors and a buoy. After 60 days exposure in the different environments, cages were to be recovered and the mussels analysed. However, due to successive storms, the cages from Ponta Grossa were lost, and only the cages from Palmas could be recovered. Therefore, the mussels transplanted to Ubatuba could not be assayed.



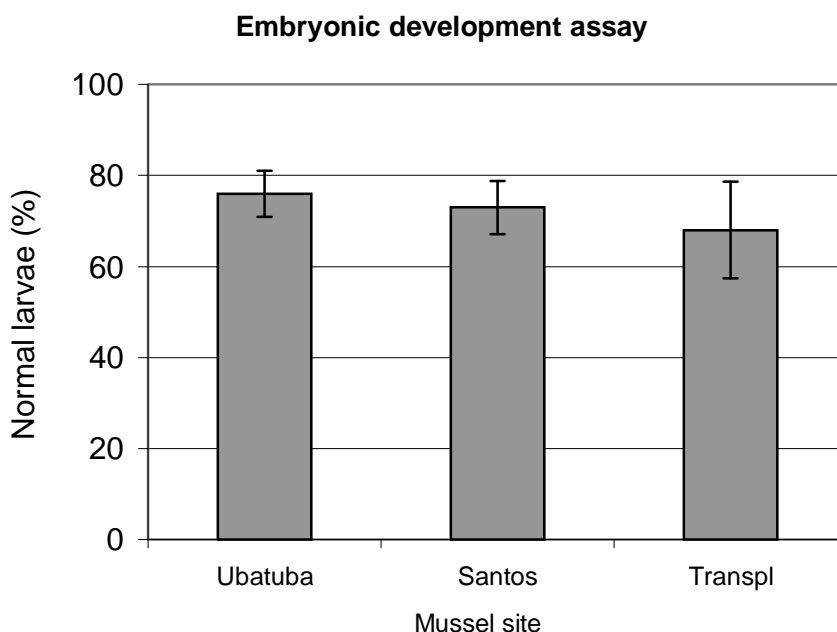
**Figure 2** - Mean NR retention times observed for each group of studied mussels.



**Figure 3** - Percent of positive response in mussel cells at each measurement, during the NR retention assay.



**Figure 4** - Mean heartbeat rate of *P. perna*, kept in clean water and at 25 °C, after 6 hours recording.



**Figure 5** - Normal embryonic development (D-Larvae) of *P. perna* after 48h.

## RESULTS

There was not any difference in the neutral red retention time between the mussels from Santos and Ubatuba, whereas the organisms transplanted to Santos showed a significantly lower retention time, which was estimated at 20.25 min (Fig. 2). Despite the absence of statistical differences, animals from Santos showed a faster response than the organisms from Ubatuba, which could be observed in the measurements made at 15 and 30 min exposure (Fig. 3).

There were no differences in the results of cardiac activity (Fig. 4). The heartbeat rate of the mussels from the three groups tested was similar, ranging from 29.30 to 32.89 beats/min.

The embryonic development was similar for the three tested groups of organisms, ranging from 68 to 76 % (Fig. 5). According to Bayne (1976), the normal embryonic development percent for mussel ranges between 70 and 100 %.

## DISCUSSION

For comparisons of the health conditions between field-collected organisms, the evaluation of different levels of organisation may provide a

more comprehensive understanding of the actual status of the studied organisms.

The cardiac monitoring method is an approach that was designed to show evidences of physiological stress in the studied animals. The heartbeat means observed for the organisms from Santos and Ubatuba and also for the mussels transplanted to Santos were very similar. A similar result was obtained for the embryonic development assay, where no differences were observed. The absence of differences can be interpreted as: 1- the organisms were exposed to similar conditions; or 2- in case of different conditions (contamination and/or environmental factors), the stress was not strong enough to cause physiological responses.

Physical-chemical studies of water from both sites show clearly that Santos and Ubatuba constitute different environments (Tab. 1), mainly due to differences in the estuarine waters influence (FUNDESPA, 1999; Frazão, 2001; Abessa, 2002; Azevedo, 2002; Moser, 2002). Thus, the results from the two methods mentioned above suggested that the organisms from both sites are not exposed to stressing conditions.

**Table 1** - Physical, chemical and biological characteristics of the water at the collection sites (NA = not analysed).

Variable	Ubatuba	Santos
Salinity (‰)	33 - 36 <sup>1</sup>	28 - 35 <sup>2,3</sup>
pH	6,9 - 8,7 <sup>1</sup>	7,9 - 8,4 <sup>2,3</sup>
Temperature (°C)	21 - 28 <sup>1</sup>	20 - 27 <sup>2,3</sup>
Dissolved Oxygen (mg/l)	5,0 - 8,2 <sup>1</sup>	5,5 - 8,1 <sup>2,3</sup>
PO <sub>4</sub> (µMol)	0,34 - 0,54 <sup>4</sup>	0,26 - 13,5 <sup>5</sup>
NH <sub>3</sub> -NH <sub>4</sub> (µMol)	0,26 - 1,09 <sup>4</sup>	0,58 - 33,55 <sup>5</sup>
NO <sub>2</sub> (µMol)	0,14 - 0,39 <sup>4</sup>	0,2 - 4,62 <sup>5</sup>
NO <sub>3</sub> (µMol)	0,10 - 0,42 <sup>4</sup>	0,6 - 31,06 <sup>5</sup>
Chlorophyl a (mg/m <sup>3</sup> )	0,64 - 1,28 <sup>4</sup>	7,3 - 97,37 <sup>5</sup>
Fecal Coliforms (NMP)	NA	100 - 500 <sup>6</sup>
Classification	Oligotrophic <sup>7</sup>	Eutrophic <sup>7</sup>

1- IOUSP, unpublished data; 2- UNISANTA, unpublished data; 3- Abessa, 2002; 4- Azevedo, 2002; 5- Moser, 2002; 6- FUNDESPA, 1999; 7- Frazão, 2001)

The use of a technique concerned with a lower level of organisation, such as the NR retention assay, provided complementary information to the understanding of the conditions of the animals at each site. The mean retention time observed for populations of Santos and Ubatuba were similar, but the transplanted mussels presented a significantly lower retention time.

It was demonstrated that environmental stressors such as higher temperatures or hyposalinity can induce lysosomal membrane destabilisation (Nicholson, 2001). Once only the transplanted organisms showed evidences of stress, the results initially suggest that the environmental conditions between Santos and Ubatuba are different, and that the transplanted mussels responded to such differences showing effects in their lysosomes. In this case, although Ubatuba and Santos constitute different natural environments, mussels inhabiting them seem to be adapted to their respective water conditions, what is evidenced by the absence of differences in the results obtained by all methods. This is supported by the previous evidences that the environmental conditions in Palmas are suitable for marine life. Abessa et al. (2002) showed that sea urchin embryos from Palmas Island, Ubatuba and other clean sites are equally sensitive to chemical substances, suggesting that the proximity of Palmas Island to contamination sources is not affecting those organisms.

However, when the percent of affected animals (given by the % organisms showing effect) in each group is compared, it is possible to see the data from a different angle. The transplanted mussels showed the fastest response, and quickly 100%

organisms showed effect. Besides, mussels from Santos tended to respond faster than the animals from Ubatuba, as could be seen at 15 and 30-min measures. This difference suggests that, although adapted to the environmental conditions found in the waters of Santos, the population from Palmas seems to be very close to the threshold of showing cellular stress.

Further investigations are recommended in order to evaluate the conditions of organisms from Palmas, once they are collected by fishermen and used for human consumption.

## ACKNOWLEDGEMENTS

This work was supported by the ODA (Overseas Development Administration). The authors thank to Dr. Guilherme Lotufo (USACE) for the first draft review and Clube de Pesca de Santos for the logistic support.

## RESUMO

Neste trabalho, foi realizado um estudo das condições fisiológicas de mexilhões de Santos e Ubatuba e também de organismos transplantados de Santos para Ubatuba, por meio de diferentes técnicas analíticas. Foram realizados testes de estabilidade lisossômica, utilizando células da hemolinfa, monitoramento da atividade cardíaca por 6 horas e ensaios de desenvolvimento embrionário, com ovos obtidos a partir de cada grupo de mexilhões. A atividade cardíaca e o

desenvolvimento embrionário foram semelhantes para os três grupos estudados. O tempo de retenção do vermelho neutro foi similar entre os animais coletados em Santos e Ubatuba, enquanto os organismos transplantados para Santos apresentaram redução no tempo de retenção do corante, indicando desestabilização das membranas lisossômicas. As diferenças possivelmente se deveram a fatores ambientais, no entanto novos estudos são necessários para confirmar esta hipótese.

## REFERENCES

- Abessa, D. M. S. (2002), *Avaliação da qualidade de sedimentos Sistema Estuarino de Santos, SP, Brasil*. Doctorate thesis. São Paulo : Universidade de São Paulo, Instituto Oceanográfico. 290 pp.
- Abessa, D. M. S.; Pereira, C. D. S.; Zaroni, L. P.; Gasparro, M. R. and Sousa, E. C. P. M. (2002), Sensibilidade de *Lytechinus variegatus*: comparação entre três populações do Estado de São Paulo. In: Congresso Brasileiro de Pesquisas Ambientais, 2., Santos. *Anais...* Santos, SP, Brazil, 3-4 July. CD-Rom, File TT32. [Paper presented].
- ASTM (1992), Standard guide for conducting static acute toxicity tests starting with embryos of four species of saltwater bivalve molluscs. E 724-89. In: *Annual book of ASTM Standards*. American Society for Testing and Materials. Philadelphia. [Section 11. Vol. 11.04].
- Azevedo, G. F. O. (2002), *Variabilidade sazonal (Outono-Primavera) da produtividade primária e biomassa fitoplânctônica na Enseada do Flamengo, Ubatuba, Litoral Norte do Estado de São Paulo*. Master of Science Dissertation. Universidade de São Paulo, Instituto Oceanográfico, São Paulo, Brazil. 159 pp.
- Bayne, B. L. (1976), *Marine mussel: their ecology and physiology*. International Biological Programme. Cambridge. 506 pp.
- Bainy, A. C. D.; Almeida, E. A.; Muller, I. C.; Ventura, E. C. and Medeiros, I. D. (2000), Biochemical responses in farmed mussel *Perna perna* transplanted to contaminated sites on Santa Catarina Island, SC, Brazil. *Marine Environmental Research*, **50**, 411-416.
- Bonetti, C. (2000), *Foraminíferos como bioindicadores do gradiente de estresse ecológico em ambientes costeiros poluídos. Estudo aplicado ao sistema estuarino de Santos - São Vicente (SP, Brasil)*. Doctorate thesis. Universidade de São Paulo, Instituto Oceanográfico. São Paulo, Brazil. 229 pp.
- Cheung, V. V.; Wedderburn, R. J. and Depledge, M. H. (1997), Molluscan lysosomal responses as a diagnostic tool for the detection of a pollution gradient in Tolo Harbor, Hong Kong. *Marine Environmental Research*, **46**, 273-241.
- Dame, R. F. (1996), *Ecology of marine bivalves - an ecosystem approach*. Marine Science Series. Boca Raton : CRC Press. 254 pp.
- Depledge, M. H. and Andersen, B. B. (1990), A computer aided physiological monitoring system for continuous, long-term recording of cardiac activity in selected invertebrates. *Comparative Biochemistry and Physiology*, **96A**, 474-477.
- Depledge, M. H. and Lundebye, A. K. (1996), Physiological monitoring of contaminant effects in individual rock crabs, *Hemigrapsus edwardsi*: the ecotoxicological significance of variability response. *Comparative Biochemistry and Physiology*, **113C**, 277-282.
- Ferreira, A. G.; Machado, A. L. S and Zalmon, I. R. (2000), Metais Pesados em moluscos no litoral norte do Rio de Janeiro. In: Espíndola, E. L. G.; Botta-Paschoal, C. M. R.; Rocha, O.; Bohrer, M. B. C. and Neto, A. L. O. (Eds.). *Ecotoxicologia: Perspectivas para o Século XXI*. Rima : São Carlos. pp. 167-182.
- Fossi, M. C.; Casini, S.; Savelli, C.; Corbelli, C.; Franchi, E.; Mattei, N.; Sanchez-Hernandez, J. C.; Corsi, I.; Bamber, S. and Depledge, M. H. (2000), Biomarkers responses at different levels of organisation in crabs (*Carcinus aestuarii*) experimentally exposed to benzo(α)pyrene. *Chemosphere*, **40**, 861-874.
- Fração, L. R. (2001), *Eutrofização por esgotos domésticos e sua relação com a ecologia e fisiologia do fitoplâncton marinho no litoral do Estado de São Paulo: Ubatuba, Praia Grande e Santos*. Master of Science Dissertation. Universidade de São Paulo, Instituto Oceanográfico. São Paulo, Brazil. 158 pp.
- Lamparelli, M. L.; Costa, M. P.; Prósperi, V. A.; Bevilacqua, J. E.; Araújo, R. P. A.; Eysink, G. G. L. and Pompéia, S. (2001), *Sistema Estuarino de Santos e São Vicente*. Technical report CETESB. São Paulo, SP, Brazil. 178 pp.
- Lowe, D. M.; Fossato, V. U. and Depledge, M. H. (1995), Contaminant induced lysosomal membrane damage in blood cells of mussels *Mytilus galloprovincialis* from Venice Lagoon: an in vitro study. *Marine Ecology Progress Series*, **129**, 189-196.
- Medeiros, P. M. (2000), *Avaliação da origem de hidrocarbonetos em sedimentos marinhos de Santos e São Sebastião, utilizando-se hidrocarbonetos marcadores geoquímicos*. Master of Science dissertation. Universidade de São Paulo, Instituto Oceanográfico. São Paulo, Brazil. 102 pp.
- Moore, M. N. (1990), Lysosomal cytochemistry in marine environmental monitoring. *Histochemistry Journal*, **22**, 189-191.



- Moser, G. A. O. (2002), *Aspectos da eutrofização no Sistema Estuarino de Santos: distribuição espaço-temporal da biomassa e produtividade primária fitoplanctônica e transporte instantâneo de sal, clorofila-a, material em suspensão e nutrientes*. Doctorate thesis. Universidade de São Paulo, Instituto Oceanográfico, São Paulo. 2 v.
- Nicholson, S. (2001), Ecocytological and toxicological responses to copper in *Perna viridis* (L.) (Bivalvia: Mytilidae) haemocyte lysosomal membranes. *Chemosphere*, **45** : (4-5), 399-407.
- Payne, J. F.; Mathieu, A.; Melvibn. W. and Fancey, L. L. (1996), Acetylcholinesterase, an old biomarker with a new future? Field trials in association with two urban rivers and a paper mill in Newfoundland. *Marine Pollution Bulletin*, **32** : (2), 225-231.
- FUNDESPA. (1999), *Levantamento oceanográfico da área diretamente afetada por efluentes de emissários submarinos de esgotos da SABESP, entre os municípios de São Sebastião e Mongaguá, Estado de São Paulo: Relatório Final*. São Paulo : Fundação de Estudos e Pesquisas Aquáticas. 2 v.
- Reish, D. J. (1987), The use of toxicity testing in marine environmental research. In: Soule, D. F. and Kleppel, G. S. (Eds.). *Marine Organisms as Indicators*. Springer-Verlag : NY. pp. 231-241.
- Rios, E. (1984), *Sea shells of Brazil*. 2<sup>nd</sup> ed. Rio Grande : Editora da FURG. 368 pp.
- Tommasi, L. R. (1979), *Considerações ecológicas sobre o Sistema Estuarino de Santos, São Paulo*. Honorary Professor Thesis. Universidade de São Paulo, Instituto Oceanográfico. São Paulo. 2 v.
- Wedderburn, J.; Mcfadzen, I.; Sanger, R. C.; Beesley, A.; Heath, M.; Hornsby, M. and Lowe, D. (2000), The field application of cellular and physiological biomarkers, in the mussel *Mytilus edulis*, in conjunction with early life stage bioassays and adult histopathology. *Marine Pollution Bulletin*, **40** : (3), 257-267.
- Wells, P. G.; Lee, K. and Blaise, C. (1998), *Microscale testing in aquatic toxicology - advances, techniques, and practice*. Boca Raton : CRC Press. 679 pp.
- Zar, J. H. (1984), *Biostatistical analysis*. Englewood Cliffs : Prentice-Hall, Inc. 718 pp.
- Zaroni, L. P. (2000), *Testes de toxicidade com a utilização de embriões do mexilhão Perna perna (Linnaeus, 1758): uma adequação do método*. Master of Science Thesis, Universidade de São Paulo, Instituto Oceanográfico, São Paulo. 47 pp.
- Zaroni, L. P.; Abessa, D. M. S.; Rachid, B. R. F. and Sousa, E. C. P. M. (2001), Diferenças no estado fisiológico de adultos e na viabilidade de embriões do mexilhão *Perna perna* provenientes de duas populações coletadas em Ubatuba - SP. In: Moraes, R.; Crapez, M.; Pfeiffer, W.; Farina, M.; Bairy, A. and Teixeira, V. *Efeitos de Poluentes sobre Organismos Marinhos*. São Paulo : Arte & Ciência Villipres. pp. 15-25.

Received: September 19, 2003;

Revised: March 23, 2004;

Accepted: December 09, 2004.