# Biomarkers for assessing benthic pollution impacts in a subtropical estuary, Mozambique

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Examinator: Professor Kristina Sundbäck, Institutionen för biologi och miljövetenskap, Göteborgs universitet.

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## Abstract

This thesis focuses on the marine environment in a subtropical estuary and particularly on exploring a suite of easy-to-use, cost effective and environmentally valid biological response tools (biomarkers). The main driving force of the thesis is the knowledge of the numerous anthropogenic impacts. Today, only scarce information is available in the Espírito Santo Estuary (ESE) in southern Mozambique on the effects of pollutants.

Links between behavioural disruption and neurological dysfunction, such as acetylcholinesterase (AChE) inhibition has been shown to be the most responsive biochemical biomarker at sites influenced by agricultural, urban and industrial activities. In **Paper I**, the enzymatic inhibition of AChE and butyrylcholineesterase (BChE) was measured in tissues (brain, liver and hepatopancreas) from barred mudskipper fish (*Periophthalmus argentilineatus*), Jarbua fish (*Terapon jarbua*), Indian white prawn (*Penaeus indicus*) and the clam *Meretrix meretrix*. In general, all species showed a significant decrease in the enzymatic activities. For *M. meretrix* the inhibition of BChE was most evident. In water of the Infulene River, tributary of ESE, 12 pesticides were found.

Besides the contaminants having a direct effect on burrowing behaviour, they also cause a sublethal physiological stress, reducing metabolic activity, growth rate and reproduction. In **Paper II**, integrated physiological assays were applied by using the scope for growth (SFG) method and condition index (CI) as tools for assessing the metabolic state in *M. meretrix* from the ESE and Incomati estuary (which is used as a comparison site)**.** Low values of SFG ($<5$ J $h^{-1}$ $g^{-1}$), as well as the low CI (that ranged from $0.80 \pm 0.02$ to $1.62 \pm 0.10$) in both investigated areas show symptoms of stress and impact of pollution as reflected in the clam *M. meretrix*. These results also show that SFG can be used as a tool to compare bioenergetic responses of clams to impacts of various environmental pollutants.

Locomotion is the most studied behaviour because it links fitness-related parameters, such as food seeking and predator avoidance and is also considered as an ecologically valuable individual biomarker of stress. In **Paper III**, the impaired burrowing activity as a signal of organismal behaviour was tested in two cross-toxicity assays using the clam *M. meretrix* (bivalvia, family Veneridae). Metals in sediments and clams were also analysed to assess the sediment quality according to international guidelines and human safety for clam consumption and. The burrowing times were different in the two experiments. This finding indicated that the metal contaminated sediments delayed the burrowing time. Based on the concentrations of metals in sediments we concluded that the northern margin of ESE is polluted principally by Cu, Ni, Cd, Co, Cr, Zn, As and Hg. As expected, sediment from the comparison site and those at the southern margin of ESE contained lower levels of these metals.

**Paper IV** presents the more ecologically relevant aspects of the adverse effects of pollutants on benthic communities in ESE and the nearby Incomati estuary. Community indicators, such as species richness, abundance were used in association with multivariate analyses of assemblage structure to assess the environmental status in three zones (i.e. the upper, middle and lower reaches) of ESE. Ecological groups and ranking of feeding strategies of benthic macrofauna were also used as bioindicators. The results showed clear differences in invertebrate density and population structure between ESE and the comparison estuary, while the three zones in ESE showed negligible differences from each other. The difference between the two estuaries was mainly due to the dominance of distinguished macrobenthic groups; polychaetes were common in ESE, while bivalves and amphipods were highly abundant.in the Incomati estuary.

It is recognized that the need of integrative tools to assess ecosystem quality is very important from a scientific point of view, but simple and pragmatic information is also necessary for stakeholders and society to get useful assessments of the impact of human pressure in the estuaries and other coastal ecosystems.

**KEYWORDS**: subtropical estuary, contaminants, biological hierarchial biomarkers, scope for growth, burrowing response, acetylcholinesterase, benthic fauna, southern Mozambique.

## Populärvetenskaplig sammanfattning

Denna avhandling fokuserar på den marina miljön i ett subtropiskt estuarium i Mozambique och studier har utförts genom användande av olika rekommenderade biologiska förenklade och kostnadseffektiva mätmetoder s.k. biomarkörer. Det undersökta området, Espírito Santo Estuary (ESE) i södra Mozambique har endast översiktligt tidigare studerats med avseende på föroreningar.
Sambanden mellan beteendeförändringar och neurologiska effekter av bekämpningsmedel på enzymerna acetylcholinesteras och butyrylcholinesteras har studerats i **arbete I** på fisk, räkor och musslor. Alla arter uppvisade påverkan i form av en minskande enzymaktivitet.

I litteraturen finns tydliga samband beskrivna mellan beteendestörningar, nedsatt metabolisk aktivitet, tillväxt- och reproduktionsstörningar. I **arbete II** undersöktes effekter på energibudgeten hos musslan *Meretrix meretrix* genom bestämning av ”scope for growth i laboratorieexperiment Dessa utfördes vid den marina forskningsstationen på Inhaca Island belägen ca.35 km från fastlandet där vattenkvaliteten är hög. Musslor från ett flertal platser i estuariet liksom från ett närbeläget förmodat rent område fraktades ut till Inhaca där försöken ägde rum. Även musslornas konditionsindex mättes. Resultaten visade att musslorna såväl från estuariet som i jämförelseområdet uppvisade stressymptom sannolikt orsakade av föroreningspåverkan.

Beteende i form av nedgrävning studerades hos musslan *Meretrix meretrix.* Eftersom beteendeförändringar generellt anses vara en av de första och dessutom känsligaste signalerna på störning har många studier utförts med bl.a. musslor. **Arbete III** behandlar inverkan på musslornas nedgrävning i sediment från olika platser i estuariet. Även musslor från olika platser i estuariet undersöktes med sediment från ett förmodat opåverkat område utanför estuariet. Resultaten visade att musslor från de norra mer förorenade delarna av ESE hade en fördröjd nedgrävning i förhållande till de från den södra mindre föroreningspåverkade sidan. Även sedimentens och musslornas innehåll av metaller mättes och förhöjda koncentrationer av Cu, Ni, Cd, Co, Cr, Zn, As and Hg kunde konstateras speciellt från den norra sidan i jämförelse med den södra.
**Arbete IV** har en mer ekologisk aspekt med studier av bottenfauna på en rad stationer i ESE samt i det närliggande Incomati-estuariet. Här undersöktes, indikatorer såsom artantal, individantal, abundans, diversitet mm. kopplat till multivariat analys för att utröna den ekologiska statusen i tre utvalda områden i ESE samt i Incomati-estuariet.
Resultaten visade på stora skillnader i täthet och populationsstruktur mellan ESE och jämförelseestuariet Incomati, medan de tre zonerna I ESE visade stora likheter. Skillnaderna mellan estuarierna utmärktes framförallt av dominansen av olika makrobentiska grupper. Havsborstmaskar var vanligare i ESE medan musslor och vissa räkarter förekom mer i Incomati- estuariet.
Det kan konstateras att det vetenskapligt finns ett behov av att finna jämförande verktyg för att bedöma ekosystemens stratus men att det även är viktigt att ge information till myndigheter, samhällsplanerare och andra befattningshavare samt till allmänheten om förorenings-situationen och dess påverkan på miljö och människa i de undersökta estuarierna samt i andra kustekosystem.

To my parents

Jorge Charles Scarlet and

Augusta Joaquim Gonçalves

## LIST OF PAPERS

**This thesis is based on the papers listed below. They are referred to in the text by their roman numerals.**

I. Sturve, J., Scarlet, M.P.J., Halling, M., Dahr, N., Kreuger, J. and Macia, A. 2015. Do pesticides inhibit nerve function in aquatic organisms in Mozambique? (Submitted).

II. Scarlet, M., Halldorsson, H. and Granmo, Å. 2015. Scope for growth and condition index in the clam *Meretrix meretix* (L.) as biomarkers of pollution in Espírito Santo Estuary, Mozambique. Regional Studies in Marine Science. http://dx.doi.org/10.1016/j.rsma.2015.03.002. I*n press*. *

III. Scarlet, M.P. J., Noor, J. G., Oghenekaro, N.O., Lisovskaja V. and Granmo, H. Åke, 2015. Burrowing behaviour response of the estuarine clam *Meretrix meretrix (L. 1758)* exposed to metal contaminated sediments from Espírito Santo Estuary. (Manuscript).

IV. Scarlet, M.P.J., Paula, J. and Gullström, M. 2015. Macrobenthic assemblage composition assessed as bioindicator of environmental status in a subtropical estuary. (Manuscript).

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### Related publications not included in thesis

Scarlet, M. and Bandeira, S. 2014. Pollution in Maputo Bay. In: Bandeira, S. and Paula, J. (Eds.). *The Maputo Bay Ecosystems*. . WIOMSA, Zanzibar Town Ch. 16, pp. 347- 371.

Abreu, D., Samussone D. and Scarlet, M.P. 2014. Heavy metal contamination of penaid shrimps from the artisanal and semi-industrial fisheries in Maputo Bay. In: Bandeira, S. and Paula, J. (Eds.). *The Maputo Bay Ecosystems*. WIOMSA, Zanzibar Town, pp. 377- 381.

A doctoral thesis at a University in Sweden can be produced as a collection of papers. The aim of the introductory part is to summarize, merge and extend the knowledge in the accompanying

## Contribution by the author of this thesis to the papers

**Paper I**. The author was involved in the planning of the field sampling and contributed substantially to the experimental work, and wrote some parts of the paper.

**Paper II.** The author was involved in the planning, collections and performance of the experiments, and wrote the paper.

**Paper III.** The author was involved in the planning, sampling and performance of experiments, and wrote the paper.

**Paper IV.** The author performed all the planning, field sampling and laboratory work and wrote the paper.

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# 1. INTRODUCTION

Worldwide, pollution in coastal areas remains a major problem due to a wide array of point and non-point pollution sources, which promotes loadings of nutrients, organic contents, chemical contaminants and pathogen borne diseases. Despite that the Northern Hemisphere presents great spotted polluted coastal areas (Fig. 1), it is expected that the increasing coastal impacts during this century will shift to the tropics and hence to many developing countries (Kenish, 2002).

Coastal areas are generally exposed to an ever increasing environmental impact due to increasing populations, urbanization, industrialization, and tourism. The negative effects of chemical contaminants on marine ecosystems in these areas require an increased knowledge of the potential effects of these impacts (Lay and Zsolnay, 1999), not least in the Southern hemisphere where less efforts have been done. The assessment of the status of critical marine habitats is a central issue in developing countries, where poverty makes coastal human populations depending on goods and services supplied by the coastal ecosystems.

Additionally, the health of an estuary depends on a combination of natural events as well as human activities. Environmental impacts by both natural events associated to global climate change and human activities are nowadays true facts in East Africa and in particular in Mozambique. Mozambique is recognized as one of the most vulnerable countries in Africa to climate change, due to its extended coastal lowlands formed by estuaries and deltas (INGC, 2009).

There is growing evidence that the waters inside Espírito Santo Estuary (ESE) in southern Mozambique are polluted by untreated sewage coming from developing infrastructure that is not connected to the existing sewage and drainage facilities and water treatment plant. However, to date there has been very little research done in this geographical area, and the majority of studies have concerned basic ecological patterns rather than the effects of specific anthropogenic inputs. The scarce or even inexistent environmental assessment, monitoring and a weak legislation in Mozambique is neglected due to diverse social demands, most of them related to natural events and population growth in inappropriate areas due to limited public funds.

The measurements of pollutants in sea water and sediments alone present some weaknesses. In water, generally low concentrations and random spatial and temporal variations occur.

Sediment bound contaminants are not always bioavailable to organisms due to their physico-chemical form and the heterogeneity of sediment particle size and organic matter contents; could make comparisons between sites difficult. Additionally, measuring contaminants in marine organisms is important from a human point of view although concentrations of pollutants do not indicate information about the toxicological significance of pollutants accumulated and do not reveal the health status of the organisms. Due to these implications actually biomarkers or biomonitors are included in field survey where they offer the potential to assess the general health of organisms and contaminated ecosystems (Galloway et al. 2004; Hagger et al. 2008).

In the present thesis the definition stated by McCarthy and Munkittrick (1996) where biomarkers are functional measures of exposure to stressors expressed at the sub-organismal, physiological or behavioural level, will be used.

## 2. AIMS

On the basis of the concerns mentioned above and the alternatives presented, the overall aim of this thesis was to assess of the usefulness of biomarkers on various biological levels to provide evidence of pollution on the ESE. To achieve this aim integrated biomarkers were used to identify the impact and levels of contaminants in some local species with ecological and economic importance for Mozambique. Thus, the specific objectives of this thesis are to:

1. Explore the use of two molecular biomarkers, AChE and BChE, on effects and exposition to organophosphorus and carbamate pesticides in different species.
2. Apply scope for growth (SFG) and condition Index (CI) as physiological biomarkers of stress at individuals of the clam *Meretrix meretrix*.
3. Test the burrowing behaviour of *M. meretrix* in relation to effects of metal concentrations in sediment and in the clam.
4. Explore the use of macrobenthic fauna assemblages, ecological groups and feeding strategy as response biomarkers of environmental status.

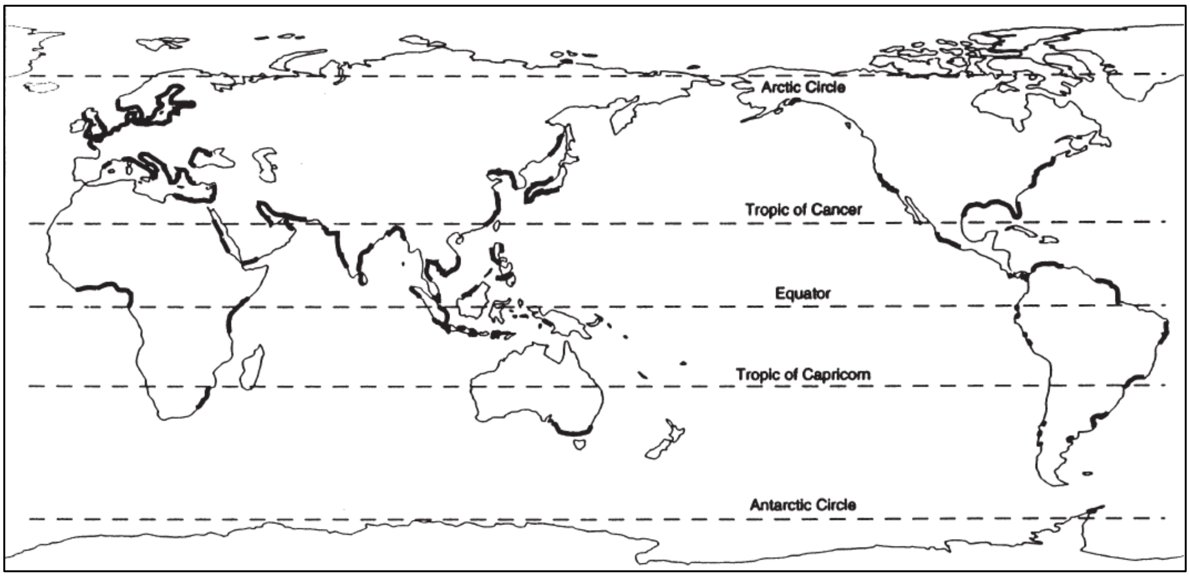


Fig. 1. World map showing existing coastal areas (in bold) significantly impacted by human activities. (Source: Alongi, D.M. (1998) Coastal Ecosystem Processes. Boca Raton, USA: CRC Press: 419 pp).

## 3. SUMMARY OF THE THESIS

The focus of this thesis is on the marine environment in a subtropical estuary and particularly to explore a suite, easy to use, cost effective and environmentally valid biological response variables or tools (biomarkers) for future successful assessment and monitoring of estuarine and coastal environments in Mozambique. Thus, some endpoints or biomarkers at different functional hierarchies were assessed for their use to evaluate the health status of ESE. The studies have been carried out through field sampling, analytical techniques, and experimental bioassays presented in the following papers.

**Paper I**

*Do pesticides inhibit nerve function in aquatic organisms in Mozambique?*

Measuring acetylcholinesterase (AChE) in blood or tissues has proven to be the most responsive biomarker at sites influenced by agricultural, urban and industrial activities (Tsangaris et al. 2010). In **Paper I**, levels of inhibition of AChE and butyrylcholinesterase (BChE) were measured in several species, including the hard clam *Meretrix meretrix* (Linnaeus, 1758), the Indian white prawn *Penaeus indicus* (H. Milne Edwards, 1837), the Barred mudskipper fish *Periophthalmus argentilineatus* (Valencienes in Cuvier and Valencienes, 1837), the Jarbua fish *Terapon jarbua* (Forsskål, 1775), and the Mozambican tilapia *Oreochromis mossambicus* (Peters 1852), which were all collected in the intertidal areas and mangroves sites in the ESE, Maputo Bay and associated rivers. Inhaca Island was selected as a reference area for all species except for *M. meretrix,* where Incomati was used as comparison site. Exposure bioassays were performed and compared to controls and the levels of 17 different pesticides were evaluated.

**Paper II**

*Scope for growth and condition index in the clam Meretrix meretix (L.) as biomarkers of pollution in Espírito Santo Estuary, Mozambique*

Contaminants in the environment cause physiological stress such as reducing metabolic activity, growth rate and reproduction. In **Paper II**, integrated physiological measurements of stress were estimated by using the scope for growth (SFG) method and condition index (CI) as tools for assessing the metabolic state in *M. meretrix.* In this study, we tested the hypothesis that the metabolism and CI of *M. meretrix* are affected by the health condition of the ESE. The response of both biomarkers are indicative of multiple stressors on the animal. Any major perturbation on the parameters of SFG must be attributed to alterations of ambient conditions such as exposure of contaminants and poor tissue condition reveal that the reserves are alternatively channelled into energy-consuming processes of detoxification or to reproduction.

**Paper III**

*Burrowing activity response of the estuarine clam Meretrix meretrix (L. 1758) exposed to metal contaminated sediment*

Locomotion is the most studied behaviour because it can be linked with fitness-related parameters such as food seeking and predator avoidance and is also considered as an ecologically valuable biomarker of stress (Little et al. 1990; Dell'Omo, 2002). Behaviour as a signal of an impaired burrowing activity was laboratory tested in two-crossed toxicity assays using the clam *M. meretrix* (bivalvia, family Veneridae) in **Paper III**. As bivalves have been addressed as sensitive indicators for monitoring of heavy metal contamination in the marine environment (Joksimovic et al. 2011; Zhao et al. 2013), levels of metals in sediments and clams were analysed. To assess whether the levels of metals in the clams were safe for human consumption, a screening-level risk assessment was conducted through a comparison of values achieved and internationally accepted guidelines.

**Paper IV**

*Macrobenthic assemblage composition assessed as bioindicator of environmental status in a subtropical estuary*

The driving environmental issue for **Paper IV** was the responses observed in the previous papers. The composition of benthic macrofauna is one of the indicated biological quality elements to be used in estuaries and coastal waters to assess environmental quality status (Dauer et al. 2008). Scarce information is available on the structure of macrobenthic fauna in the ESE. From this perspective, the paper presents ecologically realistic aspects of the adverse effects of pollutants in ESE, achieved using the composition of macrobenthic communities, sampled in their natural environment. To estimate health and environmental status, diversity indexes, benthos community structures and spatial distribution based on multivariate analyses were used. Water physicochemical parameters and sediment properties were also analysed. Because the responses to an environmental change depends on species-specific sensitivity or tolerant levels of macrofauna (Ingole et al. 2006; Chainho et al. 2006; 2007) ecological groups (EGs) were also used to assess the organic enrichment and health of the soft-bottom sediments (Grall and Glémarec, 1997). Also the feeding strategies among the benthic organisms were ranked.

## 4. BIOINDICATORS AND BIOMARKERS

*4.1 Bioindicators*

The use of bioindicators/biomonitors has been suggested to increase evidence for a significant role of marine invertebrates in assessing the impacts of environmental pollutants. Measuring pollutant concentrations in seawater presents some disadvantages such as the low concentrations and the random spatial and temporal variations. The sediment is a long-term integrator of pollution where concentrations generally are higher than in seawater, but contaminants are not always bioavailable owing to their physico-chemical forms. Moreover, heterogeneity of sediment (particle size and organic matter) could make comparisons among sites difficult. That is why the use of living organisms (bioindicators) are preferable for pollutant quantification.

A biological indicator could be defined as a species or group of species that readily reflects the abiotic and biotic state of an environment, or represents the impact of environmental change on a habitat, community or ecosystem and can be categorized as below.

i) An environmental bioindicator: represent a species or group of species that respond predictably to environmental disturbance/stress or change

ii) An ecological bioindicator: represent species known to be sensitive to pollution, habitat fragmentation or other stress.

These two groups of bioindicators have been used in the present study; **Papers II, III,** and **IV** used environmental indicators and Paper **I** was related to ecological indicators. Hence, great efforts have been done to identify the most relevant model organism to test toxicity by looking first at characteristics such as abundance, ecological and commercial importance and position in marine food chains especially for transfer of energy. The selection of bioindicators must fulfil three general characteristics that should be considered: biological relevance, methodological relevance, and societal relevance developed by Bartel (2006) and adapted from Burger and Gochfeld, (2001). The indicators selected in this thesis meet these last three requisites. Bivalves in particular mussels, have been used (e.g. the "Mussel watch programme") and considered as being sentinel organisms (Viarengo et al. 2007). Analysis of tissues of the sentinel species allows estimation of the available concentrations of the pollutant in the environment.

### *4.2 Biomarkers*

The biomarker approach, which originally was developed in pharmacology/medical toxicology (Nicholson, 2003), has recognized the importance in evaluating the adverse effects of pollutants at biological and ecosystem levels. Thus, biomarkers have become common in the field of environmental toxicology for use in ecological risk assessment and monitoring (McCarty et al. 2002).

### *Fundamental principles of biomarkers.*

Biomarkers range from general (e.g. Stress on Stress – SoS) to specific (e.g. inhibition of AChE by organophosphates or carbamates), reflecting general stress or exposure to specific environmental contaminants. The highly specific biomarkers are fewer than those which are relatively non-specific (Thain et al. 2008). According to WHO (2001), biomarkers can be classified as markers of exposure, effects, and susceptibility. In the present thesis only the biomarkers of exposure and effects were used.

*Biomarkers of exposure* are the response of integration between a xenobiotic and some target molecule or cell that is measured within a compartment of an organism. In general, biomarkers of exposure are used to predict the dose received by an individual, which can be related to a change resulting in health impairment. The fundamental principles of biomarkers of exposure are based on the principles of dose-response relationship where; i) the contaminant concentration at/in the cell or tissue is due to the biomarker response; ii) the contaminant concentration in cells/tissues can be correlated with changes in environmental concentrations of the contaminant (albeit the bioavailable fraction). According to Bartel et al. (2006), an effective biomarker of exposure should take the form of an economical and reliable biochemical or physiological measure that demonstrates bioaccumulation of a specific chemical or class of chemicals.

*Biomarkers of effects* have been defined as “ a measurable biochemical, genetic, physiological, behavioural or other alteration within an organism that, depending on the magnitude, can be recognized as associated with an established or early health impairment or disease” (Timbrell, 1998). Biomarkers of effects could provide highly specific internal measures of a detailed biological response at cellular or subcellular locations targeted by contaminants. It is often argued that since biomarker effects are measured in living organisms, the information generated is particularly useful for the protection of biological species and ultimately the management and conservation of natural ecosystems.

If the both and latter notions are satisfied in an appropriate cell tissue, or organism; then it will be a good biomarker of exposure and effect (Handy et al. 2003). In this sense, the results observed in **Papers II** and **III** show that both used methods represent the biomarker of exposure and effect.

*Issues related to the use of biomarkers*

A practical and successful biomarker should satisfy a number of criteria:

i) The biomarker response should be sensitive enough to detect early stage of the toxicity process, i.e. should precede the effect at high levels of biological organization.
ii) The biomarker should be specific to a particular contaminant or for a class of contaminants.
iii) The biomarker should respond to a concentration in the same manner as to a change in ambient levels of the contaminant.

iv) Identification of the non-toxicological variability identified in particular variations linked to biotic factors.

In practice, these four criteria are not easy to satisfy and most biomarkers have limited specificity, due for example to the variety of pollutants present in an environment. For this reason integrated biomarkers functioning at different levels of biological organization are required to be effectively applicable in a biomonitoring programme. Advantages of applying biomarkers often to complement traditional methods of detecting pollution are considerable (Wells et al. 2001; Depledge, 2009; Tsangaris et al. 2010). Some of these advantages include:

i) Biomarkers can indicate biological effects, while chemistry-based surveillance systems cannot.

ii) Biomarker responses can be used to reveal the presence of contaminants that were not suspected initially.

iii) Biomarkers indicate when a pollutant has induced a compensatory response within the organism (rather than merely being present in an inert form).

iv) Biomarker responses often persist long after a transient exposure to a contaminant that has then degraded and is no longer detectable.

v) Biomarkers analyses are in many cases much easier to perform and are considerably less expensive than a wide range of chemical analyses which require equipment and other requirements.

vi) Biomarkers approach gives a weight of evidence showing that the organisms have been exposed to or affected by contaminants.

*Variability of response*

The main causes of variability of biomarkers' response can be attributed to abiotic factors (e.g. temperature, salinity, dissolved oxygen, pH, etc.) and biotic factors (e.g. size, sex age, genotype, phenotype plasticity, tolerance, etc.). All these factors can vary both in time and space. Most of the variability of biochemical and physiological biomarkers studied rely on season, animal sex, age nutritional and reproduction status, genotype of population studied, previous exposure history and latent effects of pollutant exposure (Handy and Depledge, 1999; Van Cleef et al. 2000; Sagerup, 2002). Even if some of these factors in theory can be taken into account during the sampling design, deviations can sometimes arise in the field, e.g. lack of specimen from a certain area due to pollution and others causes. However, there is a misconception that the source of variability renders an insensitivity of the biomarker response compared to traditional chemical monitoring techniques (Handy et al. 2003). There are several possibilities that can minimize the impact of variability such as: (i) the selection of the contaminated sites as well as the reference sites within the study areas (much attention must be put on the biotic parameters and characteristics of both areas), (ii) the study design and replication of experiment, (iii) the application of multivariate techniques, and (iv) analyses of other confounding factors to reveal true pollution effects. In the present thesis, these factors of variability responses were considered.

*Sensitivity and specificity*

Due to the sensitivity of biomarkers it is generally more useful to consider multiple biomarkers designed to characterize the relationship between anthropogenic contaminant concentration, toxic damage and adverse health effects than to consider one response in isolation. Integrating the different biomarkers and bioaccumulation levels in different bioindicator species, each with a different strategy, reduces the sensitivity limitation to evaluate the stress induced by pollutants.

Biomarkers can respond to toxic stress with different degrees of specificity. Some biomarkers are highly specific; that is, they respond only to one chemical or group of chemicals. Most, biomarkers, are less specific and respond to environmental stress in general. In this thesis, the biomarkers have been used at different degrees of specificity from biochemical to population level. One of the key functions of biomarkers is to provide an early warning signal of significant biological effects and it is generally believed that sub-organismal (molecular, biochemical and physiological) responses precede those that occur at higher levels of biological organization (Fig. 2).

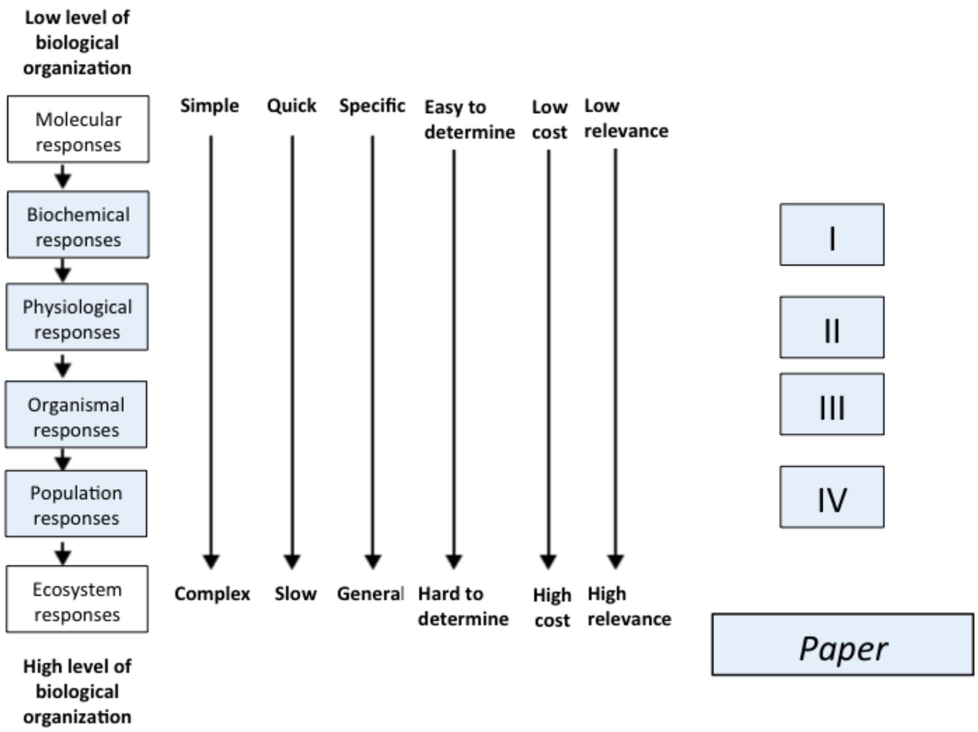


Fig. 2. Schematic illustration of biomarkers and bioindicators at various levels of biological organization used in the different papers for assessing benthic pollution impacts (the figure was adapted from Connell DW, Lam PKS, Richardson BJ, Wu RSS. Introduction to ecotoxicology. London: Blackwell Science; 1999).

*Weight of evidence in investigation and determination*

The use of biological markers or biomarkers measured at the molecular or cellular level have been proposed as sensitive 'early warning' tools for biological effect measurements in environmental quality assessments (McCarthy and Shugart, 1990). When contamination has resulted in pollution it is necessary to perform a chemical but also a biological evaluation. Since there is no single perfect tool to identify pollution, a variety of tools are required to give a weight of evidence of investigation and determination (Chapman et al. 2002). The investigation determines possible ecological impacts from chemical or other stressors based on several lines of evidence (Chapman, 2007). The determinations include both chemical and biological measurements and typically include both laboratory and field components (Chapman and Hollert, 2006). This thesis focuses on different bioindicators and biomarkers assessed by multiple techniques; laboratory assays and chemical analyses of pollutants in natural media and in organisms (Table 2 in section 6).

# 5. STUDY AREAS

Mozambique located on the southeast coast of Africa, has the third longest coastline in the continent stretching about 2,400 km and is recognized as containing the most valuable Mozambican natural resources. Due to its location the Mozambican coast is a drainage area housing more than 25 large estuaries with rivers, most of them arriving from neighbour countries where the rivers are of great ecological and economic importance. Estuaries are also the only aquatic ecosystems where the dynamic~~s~~ interaction of marine, freshwater~~s~~ and the atmosphere occurs (Kaiser et al. 2011).

The Mozambican estuaries can be classified in two major groups categorized by Whitfield (1992); those where changes are driven by global forces such as climatic and sea-level fluctuations, and those that have resulted from direct human interference and are of a local or regional nature.

This thesis focuses primarily on the poorly studied estuary in the southern part of Mozambique, the Espírito Santo Estuary (ESE) (previously named River Espírito Santo, after Maputo Estuary; e.g. Salomão, 1987), and uses the Incomati estuary as a comparison site. Both estuaries fit well into the second group categorized by Whitfield (1992).

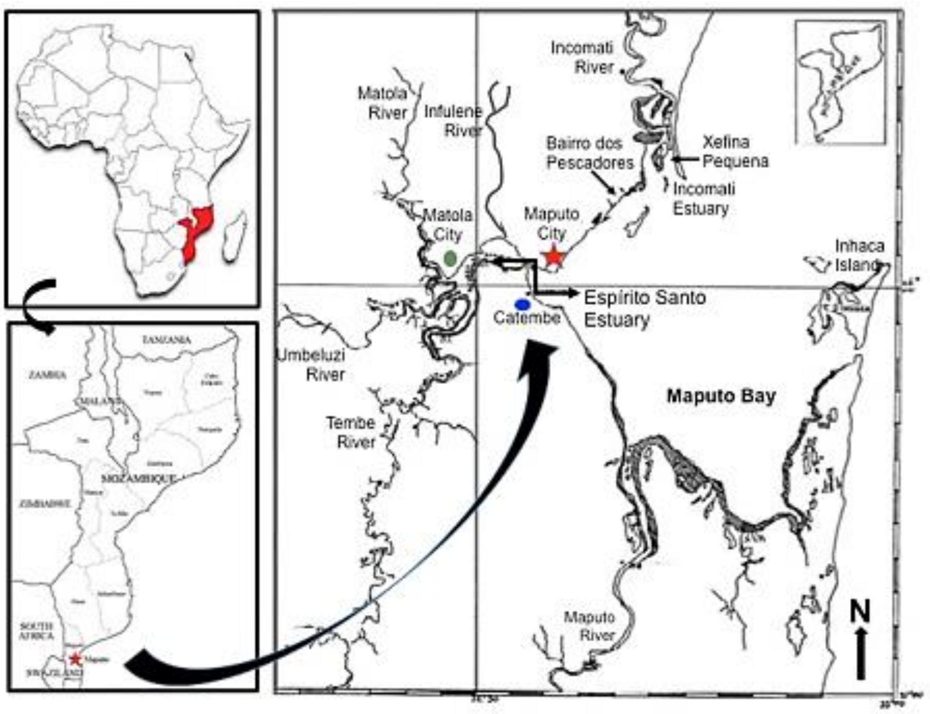


Fig. 3. Maps showing the main locations for the present.study in Espírito Santo Estuary (ESE) and the comparison site, Incomati Estuary.

*Espírito Santo Estuary (ESE)*

Espírito Santo is a socio-ecological subtropical estuary which opens into the west side of Maputo Bay at 26°S, southern coastline of Mozambique. Due to the several proposed estuarine boundaries (Elliott and McLusky, 2002; McLusky and Elliott, 2004) in this thesis the limits of ESE are the mouths of the Tembe, Umbeluzi and Matola rivers at the upstream side and a line drawn between Ponta Vermelha and Ponta Mahone, at the downstream side (Salomão, 1987) (Fig. 3). The estuary is formed in a low valley influenced by Tembe, Umbeluzi and Matola Rivers at the western side, Matola and Infulene rivers running into the northern bank of the central part of the estuary and Maputo Bay at the eastern side.

ESE, has an area of 52.53 $km^2$, 20 km in length and a maximum width of 800 meters. It is a sheltered and shallow estuary with a water depth less than 5 m over most areas with the exception of the two dredged navigation channels that are running parallel to the north margin.

The ESE experiences a sub-tropical climate characterized by two seasons; a warm and rainy (October-March) and a colder and dry (April-September) with rainfall mostly during the hot season. Maximum temperature and precipitation occur in January extending up to March. The water temperature varies from 16 °C in winter to 28 °C during summer. Differences in water temperature between surface and bottom do not indicate a real stratification as observed along the estuary. During the dry season, the salinity in the estuary is around 30-40, while it varies between 16 to 25 PSU during the rainy season. According to the vertical section of temperature and salinity the estuary can be considered a well-mixed and homogeneous estuary (Salomão, 1987). It is dominated by a semi-diurnal tide (Canhanga and Dias, 2005) and the tidal range varies from less than 30 cm and to about 380 cm during extreme spring tides. The tidal amplitude varies over the year, from about 80 cm during neap tides to about 300 cm during spring tides (Sete et al. 2002). The data is recorded by a permanent tide gauge located in the Maputo harbour (Canhanga and Dias, 2005). Maximum flow velocities in the main bed of the estuary registered in spring tides were 1.0 to 1.2 m/s (Salomão, 1987).

The shores of the EES are lined by mangroves in an area of 895 ha (De Boer, 2003). The most pristine mangroves are dense and located on the upper part of the western margin and partly of the southern bank of the estuary. The mangrove and the intertidal sand/mud beds are important nursery grounds and feeding habitats for higher trophic level species such as flamingos.

The ESE houses the Maputo and Matola cities and the two major harbours, Maputo and Matola Port. Both studied estuaries faces the similar problems to others in the region; rapid urbanization, industrial growth pressure and land reclamation. The ESE, provides ecosystem services at local, national, and international levels. The estuaries are also economically important to the life cycle and production of shrimps in Maputo Bay (Sengo et al. 2005), as clam beds (Scarlet, 2005) and as feeding areas of local and migratory avifauna (Bene, 1989). However the sustainable provisioning of ecosystem services requires broadly impacting and coastal regulatory actions based on available scientific resources. Although, despite the fact that the estuaries are being considered as delicate, complex and highly productive, other coastal ecosystems in Mozambique such as mangroves, intertidal and sea grass areas are more studied.

*Incomati estuary*

Incomati estuary is located at coordinates 25$^o$ S and 29$^o$ E. It has a funnel shape and is formed downstream the Incomati river (Fig. 3). This river, with a total length of 714 km, originates in South Africa and passes through the northern part of Swaziland and back to South Africa again before flowing into Mozambique, ending in Maputo Bay near Maputo City (Sengo et al 2005). The climate in the Incomati river basin varies according to its geography location; from cooler dry climate in the Highveld to warm and humid climate in Mozambique.

The Incomati estuary represents a unimodal flood, peaking in January-February, and hence the salinity is correlated to the freshwater flow reaching its peak at the rainy period with-salinities below 5 ppm. The temperature follows the same pattern as the salinity. The estuary is shallow and the deepest point at high water is no more than 10 m and the tides are semi-diurnal, with a maximum range at the mouth of about 3 m. The estuary is protected by the Macaneta peninsula that separates the estuary from the sea. Xefina Pequena Island and others small islands are located in the mouth of the estuary. The undisturbed part of the shore is dominated by mangroves, composed by six mangrove species strongly dominated by *Avicennia marina* (71.46%) (Macamo et al. 2014). The estuary and adjacent mangroves play a major role in the life cycles as nursery grounds for economically important fish and shellfish species as well as marine and migratory birds (Sengo et al. 2005; Hoguane, 2010). The main water uses can be seen in Table 1. The Incomati estuary and the associated Xefina Island were selected as comparison sites due to the abundance of the hard clam *M. meretrix*.

*Inhaca Island*

Inhaca Island is located on the east coast of Maputo Bay, southern Mozambique (Lat. 25°58'-26°05'S; Long. 32°55'-33°00'E) and 35 km eastward of Maputo City (Fig. 3). The island has an area of approximately 42 $km^2$ and is permanently exposed to two different hydrographical regimes; the eastern coastline facing the Indian Ocean, characterised by strong wave actions and ocean currents and the western coastline, facing Maputo Bay, which is relatively protected and with a maximum depth of 20 m (Kalk, 1995). The Island was selected as reference area due to its more pristine environment compared to the ESE.

*Limpopo River*

The Limpopo River basin draining an area of approximately 408 000 $km^2$, with a diverse landscape at the four riparian countries (Zimbabwe, Botswana, South Africa and Mozambique). The river travels a distance of over 1 750 km before it ends in the Indian Ocean, in Mozambique (see map in **Paper I**). The climate in the basin varies along the path of the river from the temperate climate of the western basin to the subtropical environment at the river mouth in Mozambique.

*4.2 Pollution in ESE*

Maputo, the capital city built on the north bank of the estuary, is nowadays facing a high pressure with a population estimated to 1,209,993 (INE, 2010). Maputo is the only city in the southern region of Mozambique with a central sewage system for collection of domestic waste into a water treatment plant (WWTP). At present, it is estimated that Maputo municipal sewage system serves only 10% of the urban population from the deteriorated functional system. The remaining untreated sewage has for a long time been discharged directly into rivers that flow into Maputo Bay through the Espírito Santo Estuary, causing pollution problems and water-borne diseases (Achimo, 2004; Spaliviero and Carimo, 2006; Scarlet and Bandeira, 2014). The WWTP is located amidst to the Infulene River valley between Maputo and Matola. The valley is one of the principal agricultural zones around Maputo City, known locally as the green belt zone (Silva and Rafael, 2014).

Microbiological pollution has been examined by Macia (1999), who documented high levels of *Streptococcus* in the urban sewage. The edible clams *M. meretrix*, from the ESE reveal high concentrations of *Salmonella* and *Escherichia coli. The* faecal coliforms (up to 60 000 per 100 g of clam tissue) exceeded the permission levels according to the European Union standards (<

300 MPN per 100 g of clam tissue) (Collin et al., 2008). V*ibrio parahaemolyticus* and other vibrios were isolated from clams in the ESE and Maputo Bay (Collin et al., 2012). *V. parahaemolyticus* has been associated with diarrhoea in Mozambique, (Ansaruzzaman et al. 2005). Other bacteria such as Aeromonas, Pseudomonas, Pasteurella, Grimontia and Shewanella species were also isolated (Collin et al 2012). Untreated sewage discharges are the main causes of these results and constitute a public health risk, particularly for shellfish consumers.

The main industrial sector in Mozambique is located in the Maputo and Matola (a satellite city of Maputo) situated close to the ESE. Industries located nearby, such as the MOZAL and Mozambique Cement factory, are also believed to contribute to an impact of aerosols and air smoke in the estuary (Broeg et al. 2008; Queface, 2014).

The use of pesticides in Mozambique is a practice related to the agriculture but is mainly used in healthy programmes as malaria vector control through outdoor or indoor residual spraying (IRS) and was incrementally introduced in the southern part of Mozambique (Casimiro et al. 2007). Recently, high levels of pesticides such as DDx (compounds of dichlorodiphenyl trichloroethane – DDT) have been detected at two sites in the ESE (CSIR, 2014). Additionally, increased levels of PAHs and also polychlorinated biphenyls (PCBs) were found. Research in the ESE has earlier been done to evaluate the levels of metal contamination in estuarine fish, crustaceans and bivalves, as well as in sediments (Hall and Valente, 1974; Fernandes, 1996; Maia, 1999; Achimo, 2002; Böhlmark, 2003; Svard, 2004; Mahumana, 2010). The estuary is prone to pollution due to its location, influence of tributary rivers, surroundings human settings and activities (Table 1). For more detailed information see Scarlet and Bandeira (2014).

The data presented show that the attention paid to pollution of ESE is not new and it is a constant and a very important concern. Lack of resources for adequate environment monitoring and management are the main reasons for increased pollution in the estuary, and consequently also in Maputo Bay.

Table 1. Activities linked to the tributary rivers of the Espírito Santo Estuary.

| Rivers | Countries | Activities | Reference |
|---|---|---|---|
| Umbeluzi | Swaziland<br>Mozambique | Commercial agriculture (mainly sugarcane but also fruits: citrus, banana, macadamia nuts)<br>Livestock and goats keeping<br>Agro-based industry (sugar mills)<br>Untreated wastewater (treatment plants in greater Maputo)<br>Small scale farming (along the river and in greater Maputo)<br>Hydropower generation (2 dams in Swaziland; 1 dam in Mozambique)<br>Water abstraction for Matola and Maputo cities | Carmo Vaz and van der Zaag, 2003<br>Nkomo and van vander Zaag, 2004<br>Gustafsson and Johansson, 2006<br>Sengo et at. 2005<br>Hoguane, 2010 |
| Matola | Mozambique | Beluluane Industrial Park (metalworking; forging; steel pipes production; textiles manufacture; production and repair of hydraulic and pneumatic measuring instruments; aluminium smelter and aluminium parts production; processing and packaging of cashew nuts; rubber components production; cement plant; storage, filling, transport and distribution of domestic gas; recycling and processing of waste oils for lubricants and plastic production; tires retreating; Pepsi production) | Tembe and Baloi, 2001<br>Sola, 2010<br>Sumalgy, 2011<br>Scarlet and Bandeira, 2014 |
| Infulene | Mozambique | Maputo water treatment plant<br>Untreated waste water (domestic and industry; alcoholic drinks production – Gin, Whisky, Rum, Beer; paper production)<br>Small scale farming (vegetables and fruits; mainly maize, cassava, groundnuts and cowpeas) | Van Burren and van der Heide, 1995<br>Chibantão, 2012<br>Scarlet and Bandeira, 2014 |
| Tembe | South Africa<br>Mozambique | Agricultural use, deforestation due to rampant logging, fires, extensive livestock cattle and goats | Macia, 2009 |

# 6. METHODOLOGY

*6.1. Sentinel organisms or bioindicators used*

This thesis included some representative species found in the ESE, Maputo Bay and some rivers (indicated above), as bioindicators or sentinel organisms, such as:

1. *Meretrix meretrix* (Linneaus, 1758) – Hard clam

This clam is an invasive species commonly found in the inner parts of the western side of Maputo Bay. The species is sedentary and filter-feeding which favour bioaccumulation of contaminants. It has been used for assessment of water quality, such as municipal treatment effluents (Wan et al. 2015) and microbiological contamination (Nenonen et al. 2006; Collin et al. 2008; Collin et al. 2012). This clam is particularly important as food in coastal areas near Maputo, mostly because it is easily collected in shallow areas and have high nutritional value, and as in many countries (e.g. China, Japan, Vietnam and India) a clam aquaculture takes place in the centre of Mozambique.

2. *Penaeus indicus* (H. Milne Edwards, 1837) – White prawn

This prawn species is easy to catch as it seldom buries in the mud. Specimens are found in inshore and estuarine waters such as in the mangroves were they settle during their post-larval stages after spawning in the sea (Rönnbäck et al. 2002). Prawns are one of Mozambique's most important exported seafood and together with fish also a main part of the local diet (Chemane et al. 1997). They are fished by traps in the ESE.

3. *Peryophtalmus argentilineatus* (Valenciannes in Cuvier and Valenciannes, 1837) – Barred mudskipper.

This is a stationary species in the mangroves. Due to its amphibious life style they can dwell in the intertidal zone, an environment with highly variable conditions, without competition from other fish species (Kruitwagen et al. 2007). The mudskipper has been used in previous studies as a sentinel species for monitoring purposes of marine pollution (Bu-Olayan and Thomas, 2008).

4. *Terapua jarbua* (Forsskål, 1775) – Terapon fish or thornfish

Terapon fish are common in most mangrove areas (Nagelkerken, 2008) and like many other fish species the omnivorous *T. Jarbua* spends the juvenile life stage in the mangrove and return to the sea to spawn (Allen, 1991).

5. *Oreochromis mossambicus* (Peters, 1852) – *Mozambican tilapia*

This fish is native to Africa, found today in subtropical and tropical fresh waters all around the world due to aquaculture (Canonico et al. 2005). The species has been used extensively for toxicological studies and is commonly found in the fresh water systems in Mozambique.

### 6.2. *General Methodology*

Several methods have been used within the scope of this thesis. The methods applied are exclusive to each paper or study. Detailed descriptions of each method are referred to in each paper and a summary is found in Table 2. Most of the methods and techniques have been used in earlier studies, although they are new for subtropical environments and in particular in East Africa.

Furthermore, as Mozambique has no specific regulatory guidelines for coastal waters it was necessary to adopt international guidelines for comparisons.

Most of the measurements, bioassays and experiments were done at the Maritime Biological Station of Inhaca (EBMI) at Inhaca Island due to its high seawater quality.

Table 2. Summary of bioindicators, biomarkers, methodology, parameters studied and treatments used in **Papers I-IV**.

| Paper | **Bioindicator** | **Biomarker** | **Methodology** | **Parameters studied/analysed** | **Treatment** |
|---|---|---|---|---|---|
| **I** | *Meretrix meretrix*<br>*Periophtalmus argentilineatus*)<br>*Terapon jarbua*<br>*Penaeus indicus*<br>*Oreochromis mossambicus* | Biochemical response<br>Acetylcholinesterase (AChE)<br>Butyrylcholinesteease (BChE) | Experimental spectrophotometric measurements<br>Experimental exposure (48 –h) to pesticides.<br>Solid phase extraction (SPE) in water using Biotage | Enzymatic activity AChE and BChE – organophosphates and carbamate pesticides<br>Fish:<br>- Liver<br>- Brain<br>Clam and Prawn:<br>- Hepatopancreas<br>Exposure – Malathion and Diazinon pesticides | One way ANOVA-(SPSS v. 22) |
| **II** | *Meretrix meretrix (hard clam)* | Physiological response<br>Scope for growth (SFG)<br>Condition index (CI) | Experimental Measurements and calculations | Clearance rate<br>Respiration rate<br>Energy consumed<br>Energy lost<br>SFG<br>CI (shell size and meat weight) | One way ANOVA-(SPSS v. 19) |
| **III** | *Meretrix meretrix (hard clam)* | Behavioural response<br>Locomotion (burrowing) | Two short term experiments (24-h)<br>Analytical measurements | Total clams burrowed<br>Time of burrowing (ET50)<br>Metal concentrations in:<br>- sediments<br>- clams<br>Sediment grain composition | Kaplan-Meier estimates<br>Log-rank test for pair-wise comparisons(R v. 3.1.1 )<br>Cluster Analysis (PRIMER v. 5) |
| **IV** | Biodiversity | Population response | Experimental Measurements<br>Multivariate analysis | Physicochemical environmental parameter<br>Sediments proprieties<br>Benthos community structure (cumulative dominance, diversity indices)<br>Benthos spatial structure<br>Ecological groups indicators of organic enrichment<br>Feeding strategy | Multivariate analysis -(Primer package v. 6)<br>Analysis of variance<br>One way ANOVA and One way ANOSIM -(SPSS v. 22) |

### 6.2.1. *Biochemical response*

Acetylcholinesterase (AChE) and butyrylcholine esterase (BChE) activity was measured in hepatopancreas tissue in prawns and clams and brain and liver tissue in fish (**Paper I)**. The enzymatic activity inhibition was assayed following the method by Ellman et al. (1961), adapted to microplate reading. Total protein content in tissues was determined by the modified method of Lowry et al. (1951) and bovine serum albumin (BSA) was used as a protein standard. BChE was measured as described above using butyrylthiocholine as substrate instead of acethylthiocoline. Freshwater samples for pesticide analyses were collected and the pesticides were extracted using Isolute C-18 columns (Biotage), which adsorbs hydrophobic and semi-hydrophobic substances. The rational of water samples was to get an initial understanding of the classes and levels of pesticides present in the aquatic systems studied. Exposure experiments (48 h) to Malathion and Diazinon, were also conducted using white prawns and barred mudskippers.

### *6.2.2. Physiological response*

In **Paper II**, physiological biomarkers including scope for growth (SFG) and condition index (CI) were measured in *Meretrix meretrix,* sampled at different locations of ESE and the comparison site, Inhaca Island. The methodology used for SFG described by Widdows and Staff (2006). SFG was used to provide an integrated measure of *M. meretrix,* based on energy available for various functions including growth and reproduction. The CI was estimated according to Riascos et al. (2012). Due to scarce facilities to cultivate algae baker's yeast, *Saccharomyces cerevisiae*, was used after heated to 60–70 °C in hot water bath. Allometric relationships were also analysed. The clams were acclimated (3 days) in sea water before the measurements were performed at Inhaca Island.

### *6.2.3. Individual response*

Individuals of the clam *M. meretix* from the northern and southern locations of ESE were hand collected in the intertidal area during low tide together with sediment and transported to EBMI. Two short-term bioassay tests were then carried out using natural sediments (Fig. 4; **Paper III)**. Five boxes per location were left overnight to stabilize and the burrowing test started 15 hours later after a careful water aeration. In each replicate 8 specimens of *M. meretrix* were placed on the surface of the sediment. The numbers of burrowed clams were recorded according the method adapted from Phelps et al. (1983). Afterwards the concentrations of metals, were analysed in sediments and in the clams. Sediments grain size composition were examined according to Buchanan and Kain (1984).

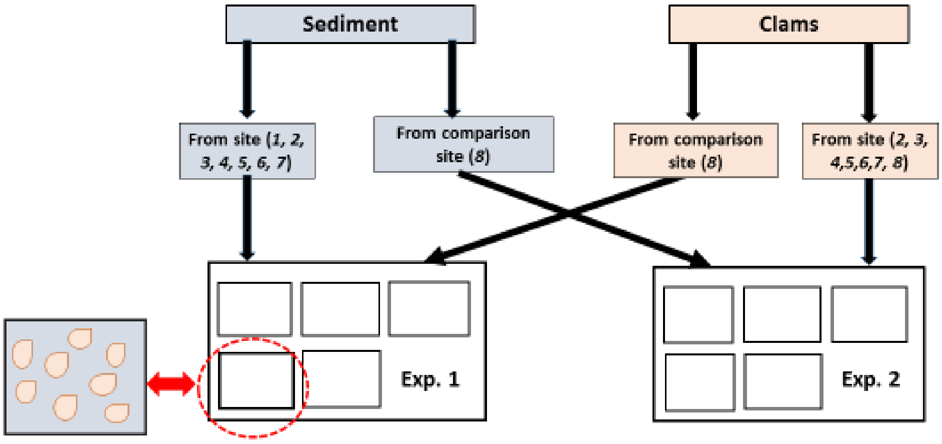


Fig. 4. Scheme of the short-term bioassay (24 h) designed to assessing burrowing behaviour in the clam *M. meretrix*. In Exp. 1, sediment collected from estuarine sites were combined with clams from site 8, the "comparison" site and in Exp**.** 2**,** sediment collected from the "comparison" site combined with clams from the estuarine and the "comparison" sites.

### *6. 2.4. Population responses*

In **Paper IV**, the ESE was divided into three zones designated as upper (U), middle (M) and lower (L) zones, based on shore characterization. Incomati estuary was used as a comparison zone. Macrobenthic fauna were sampled at each subsite (n= 7) in triplicate, within the four locations and by using a hand van Veen grab (0.025 $m^2$). Environmental variables were measured near the surface (at 0.5 m depth). The macrobenthic assemblage's structure were tested by multivariate analysis. Species abundance and diversity were also measured. Sediments sampled were used for analyses of grain size and total organic carbon (TOC). From a plethora of methodologies with several metric indices and available evaluation tools (Borja and Dauer, 2008; Diaz et al. 2004) we separated all taxa into ecological groups and classified them based on Borja et al. (2000), Bigot et al. (2008), Shokat et al. (2010) and Forde et al. (2013) in order to get an insight on the enrichment organic content in the sediments. Feeding strategy was classified according to Froján et al. (2005).

## 7. MAIN FINDINGS AND CONCLUSIONS

As presented in Fig. 2, the knowledge of the level of biological organization is important for understanding the linkage between contaminants, and their effects on different marine ecosystems. Among different biological effects of contaminants or stressors, biochemical responses occur more quickly and before other toxicological endpoints, including death (Livingstone, 1998). Despite that these biomarker responses are difficult to interpret at an ecologically significant level, many of them are easy to perform and provide an early warning signal. Thus, they can serve as a base for application of other biomarkers' responses at higher levels of biological organization.

In this sense, and according to the knowledge of pollution and concern about the contaminants in the estuary, I started my investigations at the lower level of biological organization, proceeding to the higher levels. Basic information of pesticides used nearby the Espírito Santo estuary was obtained from authorities. The results in **Paper I** showed that the field study of the AChE and BChE activity, measured in tissues of several bioindicators, including hepatopancreas of *Meretrix meretrix* (hard clam) and *Penaues indicus* (white prawn) and liver and brain in the fish species *Peryophthalmus argentilineates* (barred mudskipper) and *Terapua jarbua* (jarbua fish), varied among species as well as among locations. However, the AChE and BChE activities were generally lower in ESE than at the comparison sites meaning that an inhibition of enzyme activity occurred. Significantly lower BChE activities were observed for both the clams and prawns compared to the comparison sites (Fig. 5). These results suggested that the BChE is more sensitive than the AChE in the clam and prawn species investigated. A decreased activity of AChE in the brain was observed in barred mudskipper collected from L. Cabral but not in those collected at B. dos Pescadores. However in Jarbua fish from the L. Cabral site, the AChE activity was higher compared to the comparison site. Laboratory exposure studies to organophosphates (Ops) shows that Malathion and Diazinon in mudskipper fish had a more evident dose response effect with decreasing AChE activities compared to the white prawn. These results are in accordance with several laboratory studies on the effects of organophosphates in aquatic organisms (Brandao et al. 2013; Jordaan et al. 2013; Mahboob et al. 2014).

On the basis of the findings from field and experimental exposure studies, we concluded that the inhibition of AChE activity is a good biomarker of neurotoxicity caused by exposure to organophosphate and carbamate pesticides and that the selected species showed to be good indicators of organophosphates and carbamates pesticides. The same conclusions were stated previously by several authors (e.g. Fulton and Key, 2001; Valbonesi et al. 2003; Lau et al. 2004; Jordan et al. 2013). The analysis of BChE activity in parallel with AChE activity showed to be a supportive measurement, although the findings achieved on *Meretrix meretrix* in particular should be studied further.

Apart from insecticides, a few other contaminants, including cadmium, mercury, lead and copper have been found to induce effects on anticholinesterase activity (Frasco et al. 2005; Lionetto et al. 2013). Other studies suggest that the decreased AChE may also indicate a general stress (Lehtonen et al. 2006). Despite this finding, the levels of pesticides found in water (**Paper I**, Table 3) suggested that the environment is contaminated.

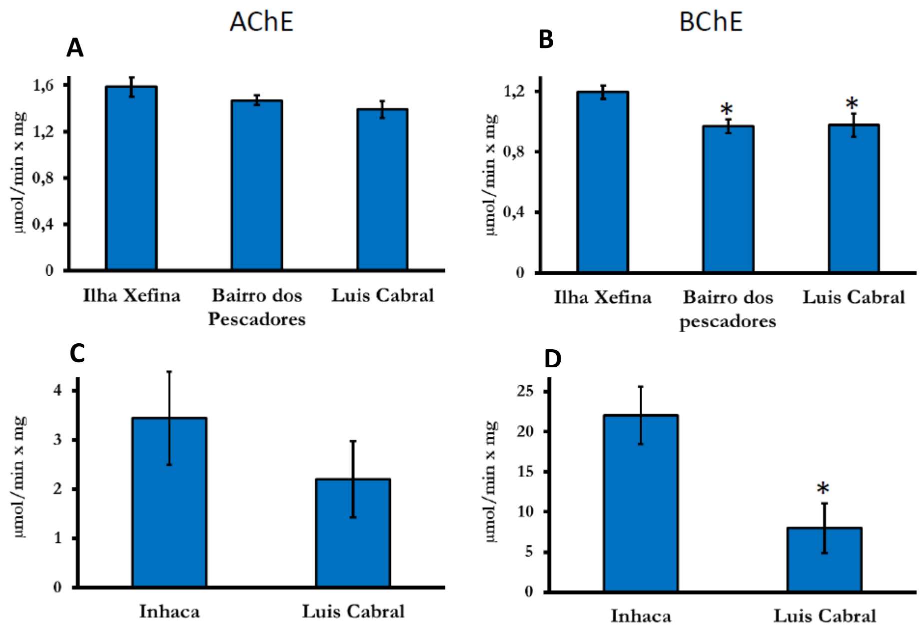


Fig. 5. AChE and BChE activities in hepatopancreas of shrimp and clam collected at different sites in Maputo Bay, Mozambique. A) AChE activity in clam (n=10); B) BChE activity clam (n=10); C) AChE activity in shrimp (n=10-13); and D) BChE activity in shrimp (n=10-13). * indicates a significant difference ($p<0.05$) between the main sites in Maputo Bay (Bairro dos Pescadores and/or Luís Cabral) and the reference sites at Inhaca Island (shrimp) or Ilha Xefina (clam). Values are given as mean ±SE.

The role of biochemical biomarkers as early warning tools is recognised as difficult to understand but changes in physiological systems (e.g. sensory, hormonal, neurological and metabolic) and fitness seem to be common responses in marine organisms exposed to stressful pollutants (Alquezar et al. 2006; Faucher et al. 2008). Many parameters have been investigated to assess disturbances of various physiological functions linked to chemical exposure (Van der Oost et al. 2003). In the present thesis **(Paper II),** scope for growth (SFG) and condition index (CI), used as physiological biomarkers in *Meretrix meretrix*, showed lower, but not significantly different SFG rate values for clams from different sampling sites and the comparison site. The values achieved of SFG were <5 J $h^{-1}$ $g^{-1}$ and the CI ranged from 0.80 ±

0.02 to 1.62 ± 0.10 (Fig. 6). These low rates indicated by the physiological measurements seemed primarily to result from the low rates at which the individual clams filtered the water. However, SFG rate values from clams collected at sampling sites located in the inlet mouth at the southern bank of the estuary were slightly higher, but not significantly different compared to the comparison site. Low values (i.e. < 5 J $g^{-1}$ $h^{-1}$) were reported for higher levels of pollution stress and poor water quality in other bivalve species such as the mussels *Mytilus galloprovincialis* (Widdows et al. 1997) and *Mytilus edulis* (Widdows et al. 2002). Our results suggested that areas in the northern margin of ESE and areas surrounding Xefina Island show symptoms of stress and impact of pollution. Furthermore, SFG can be used as a tool to compare bioenergetic responses of clams to impacts of various environmental pollutants. *Meretrix meretrix* was found to be a suitable candidate species for this test.

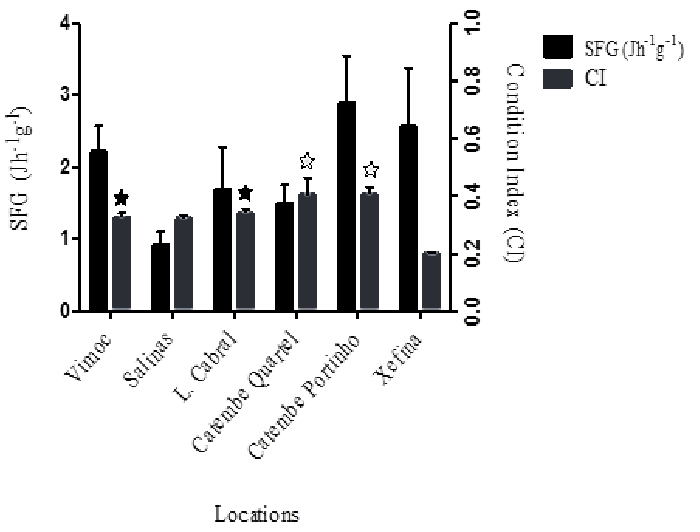


Fig. 6. Scope for growth (SFG) (Mean ± SE, n=10) and condition index (CI) in *M. meretrix* from the sampling sites and the comparison site Xefina. Symbols in the columns indicate statistical differences ★ p<0.05 and ☆ p<0.001, respectively, as analyzed by one-way ANOVA.

If the amount of toxic chemicals accumulated in the organisms reaches levels above where normal homeostatic capacity or repair mechanisms are disturbed, deleterious effects might be observed at the individual level. Since fitness is reflected in an animal's behaviour, this organismal biomarker will provide a link between the integration of physiological systems and environmental conditions. Thus, in order to integrate the two previous biochemical and physiological biomarkers applied in *M. meretrix*, **Paper III** presents results of locomotion behaviour in bivalves as the burrowing time investigated in a short sediment toxicity test. The results showed that the mean (percent) number of clams that burrowed during the 24 h bioassay ranged from 52.5% to 67.5% in Exp. 1, and from 82.5% to 95% in Exp. 2 (Fig.7). Log-rank test showed significant differences ($p<0.001$) between burrowing profiles. For Exp. 1, Cat. Portinho differs clearly from the others. This was also observed in the second experiment, where Cat. Portinho seems to differ substantially from other sites, with the exception of L. Cabral. These results showed that the burrowing by *M. meretrix* was influenced by the levels of metals measured in sediments and in contrast to the results from the second experiment, where the levels of measured metals in the soft tissues did not seem to affect the burrowing behaviour. The present study demonstrated that high metal content in the sediment tends to cause hypoactivity in the burrowing responses of the clams. This finding is also confirmed by previous studies, mainly due to the effect of Cu and Cd (Phelps et al. 1983; Byrne and O'Halloran, 1999; Shin et al. 2002; Riba et al. 2004; Bonnard et al. 2009). Also, links between behavioural disruptions and neurological dysfunctions through AChE inhibition are well-documented for aquatic biota (Amiard-Triquet, 2009, and this study). The burrowing times of the clams from the sites located at the northern margin of the estuary were markedly different as compared to clams from the southern margin and the comparison site, Xefina. Metal concentrations in the sediments achieved by hierarchical cluster analysis also reveal a difference between northern (A) and southern (B) clusters (Fig 8). Comparison of metal concentrations with sediment quality criteria showed that the sediments from the northern margin are contaminated mainly by Cr, Ni and Cu. High levels of Cu, Cd, Zn and Cr in *M. meretrix* exceed maximum permissible levels. We conclude that the burrowing behaviour of *M. meretrix* has shown to be a sensitive and useful bioassay for studying the impact of contaminants in marine ecosystems.

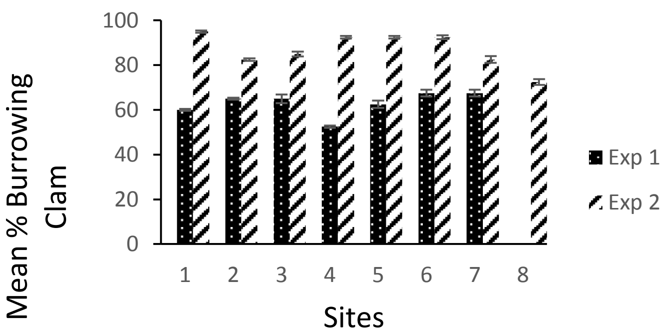


Fig. 7. Histogram showing mean % of burrowed clams (± SD) in in tested and control sediments in Exp. 1 and Exp. 2. The sites are represented by numbers (1-Empazol, 2-Vimoc, 3-Salinas, 4-Luís Cabral, 5-Catembe Quartel, 6-Catembe Portinho, 7 -B. Pescadores and 8-Xefina Pequena (comparison site))

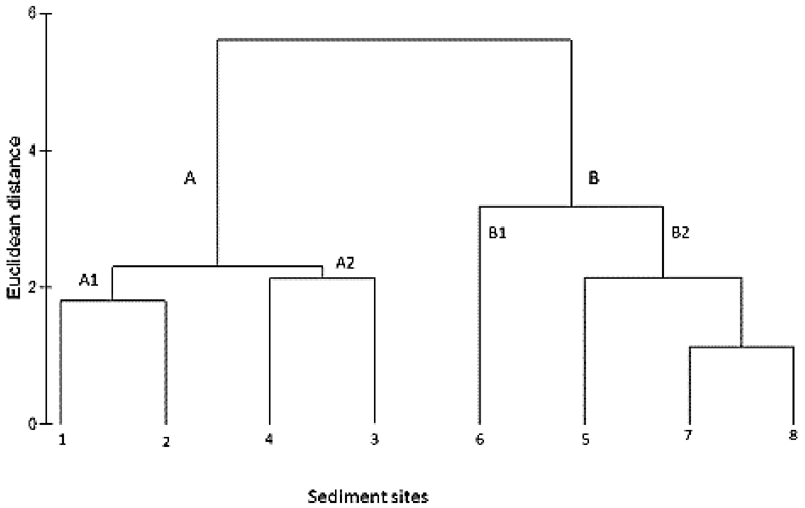


Fig. 8. Classification of all field sediments (Emp = Empasol, Vim=Vimoc, L.Cab =Luís Cabral, Sal= Salinas, Cat. Pot= Catembe Portinho, Cat. Qua- Catembe Quartel, B. Pes- Bairro dos Pescadores, Xef – Xefina Pequena), using Euclidean distance as a correlation measure of metal content. Cluster A containing sediment samples from the northern margin and cluster B from the southern margin. See legends in Fig. 7 for site numbers.

Referring to the statement by Adams (2003) that if a sufficient number of organisms are impacted, the response to stress might be subsequently measured as changes in population size or alterations in community structure, the results from the field study described in **Paper IV** showed clear differences in invertebrate density and assemblage structure between ESE and the comparison site (Incomati), while the three selected zones (upper, middle and lower) in ESE showed negligible differences from each other. The difference between the two compared estuaries was mainly due to the dominance of certain macrobenthic groups. For instance, polychaetes, were common in ESE, while amphipods and bivalves were highly abundant in Incomati (Fig. 9). The comparison sites showed a wide range of physico-chemical characteristics. On the basis of these results and the studies at lower levels of organization, it could be assumed that variation in assemblage structure of the benthic organisms observed at the four locations (the three zones of ESE and Incomati) is driven by multiple natural and anthropogenic environmental factors. On the other hand, the results reflect an unbalanced healthy benthic community compared to other estuaries. The amphipod genus *Corophium* and the polychaete genus *Nereis* found in the middle part of ESE and in Incomati are members of Group III (according to Borja, 2000 and Shokat et al. 2010) characterized as tolerant species to excess of organic matter enrichment indicating a slight unbalanced situation. The polychaete *Cirratulus cirratus* found in the upper part of the ESE is categorized as belonging to Group IV, is an opportunistic species indicating a slight to pronounced unbalanced situation. This study suggests that assemblage composition of benthos and the preliminary ecological groups found may also – to some degree – be useful as bioindicators. Further studies on the ecological group's identification are required.

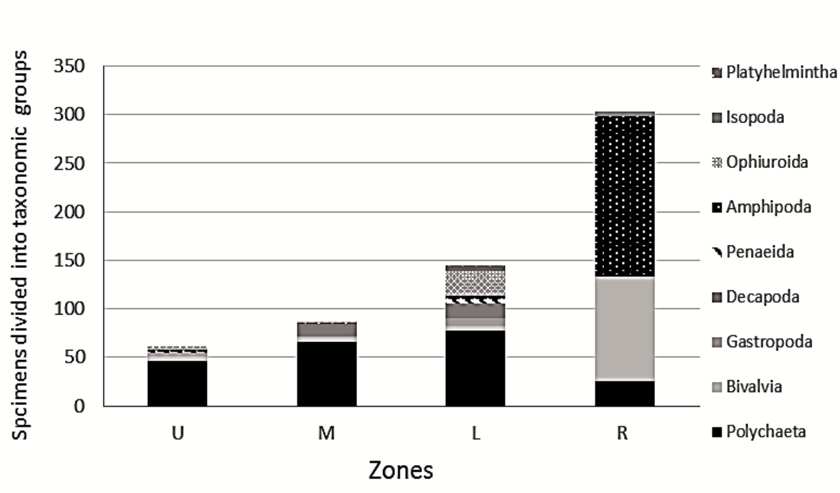


Fig. 9. Total number of taxonomic groups of macrobenthic fauna collected and identified at the three divided zones (U – upper, M – Middle, L – Lower) at ESE and Incomati estuary as a comparison site (R).

To summarise, this thesis constitutes an attempt to bring together some research methods and tools. The studies performed in this thesis are the first to investigate the effects of pollution and the relationship between sediment, pollution and benthic fauna in the ESE.

All hierarchical levels of biological response applied in this thesis meant to identify the mechanistic linkage between the lower level of response, e.g. biochemical biomarkers, and the higher relevant levels, such as responses on population and community levels, were estimated. The biological approach used here through field and exposure methodology, in combination with chemical measurements of contaminants (pesticides and metals), provided a validation of the biomarkers and also confirmed the hypothesis of a stressed estuary. It is recognized that the need for integrative tools to assess ecosystem quality is very important from a scientific point of view, while also simple and pragmatic information is necessary in order to show stakeholders and society proper assessments the impact of human pressure in estuaries and other coastal ecosystems.

Regarding responses of the biomarkers tested in this thesis some concluding remarks can be drawn:

i) All biomarkers used in this thesis were found suitable to be used for studying the impact of pollutants in the ESE.

ii) Some of the macrofaunal species used are suitable to apply in routine biomonitoring programmes in the subtropical region of Mozambique. *Meretrix meretrix*, however, is not found in the central and northern parts of the country,

iii) A chemical–biological approach is necessary to facilitate assessment of the anthropogenic pressure and also to better understand any environmental effects,

iv) Investigations at a low level of biological organization were found to be valuable, but must be framed in the integrated response to give a better ecological relevance,

v) The research highlights the effects of contaminants associated to the benthic fauna (*M. meretrix* and *Penaeus indicus*) that can affect the public health (natural resources catches in the ESE) in the principal cities of the Maputo and Matola.

vi) The thesis is to our knowledge the first to investigate the use of a whole suite of biomarkers at different biological levels of organisation in Mozambique.

The usefulness of any biological-effect method will obviously depend on how well anthropogenic stressors can be separated from the influence of environmental or host related processes. This could partly be solved by using a suite of different biomarkers which respond in different ways to environmental disturbances, i.e. "*more eyes see better*".

The study notes that contamination of natural resources in the ESE is caused by several disruption factors such as continued sewage and industrial wastewater discharge without treatment in tributary rivers that end in the ESE, nearby agriculture and pesticide use, mangrove deforestation for salt production, estuary dredging, urbanisation and coastal slope erosion. Thus, I am convinced that pollution management in the ESE is a complex but a challenge to tackle.

## 8. FUTURE CHALLENGES

Some uncertain or variability factors in the present studies must, however, be considered as challenges for future research. It is necessary to identify better reference sites (pristine if possible) to analyse the response of biomarkers for a given ecological system. Although the selected biomarkers are best suited for the objectives of this thesis, there is a need for further testing of other simple biomarkers at different biological levels (e.g. lysosome membrane stability [LMS], stress on stress [SoS] and DNA unwinding) and to find other sentinel species to use. To get a weight-of-evidence, the experiments must be based not only on sensitive short-term responses but also longer-term responses must be considered.

The physico-chemical environmental processes are different at different latitudes as also the composition of biota as a result of diverging evolutionary processes in tropical and temperate coastal zones. It is also reasonable to assume that the biota ability to successfully respond to an anthropogenic input will also be affected by the different physical environments.

The information and data generated are likely to influence future studies in the area. The study has shown that there is need now, more than ever before, to apply integrated studies and to test several other biomarkers. Also more concerted communications must be established between the university, stakeholders and private companies about sources and state of pollution in the study area. Actions should then be taken to increase environmental quality and ensure that local and vulnerable people are to be guaranteed a safe consumption of fish, clams and prawns.

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